REMARKS

Claims 1-29 are pending in the subject application.

The December 11, 2009 Interview Summary

Applicants thank Examiner Audet for the courtesy extended in conducting the telephonic Examiner Interview held on December 11, 2009 between the Examiner and the undersigned. During the December 11, 2009 interview, the rejection of claims 1-29 as allegedly unpatentable under 35 U.S.C. 103(a) over Szabo et al. (U.S. 6,531,464) in view of Arnon et al. (U.S. 6,214,791) and Kerwar et al. (U.S. 4,617,319) was discussed. Applicants' representative presented arguments substantially as set forth below. The Examiner agreed to reconsider the rejection in view of these arguments.

Claim Rejections - 35 USC § 103(a)

In the September 24, 2009 Final Office Action, the Examiner maintained the rejection of claims 1-29 as allegedly unpatentable under 35 U.S.C. § 103(a) over Szabo et al. (U.S. Patent No. 6,531,464) in view of Arnon et al. (U.S. Patent No. 6,214,791) and Kerwar et al. (U.S. Patent No. 4,617,319).

Specifically, the Examiner maintained that Szabo et al. disclose applicants' claimed combination of glatiramer acetate and mitoxantrone by virtue of the list of five multiple sclerosis-agents in claims 10 and 11.

The Examiner also asserted one of ordinary skill in the art could presume that using two known compounds can readily be used to treat multiple sclerosis, in combination, and that one would be expected to enhance the other.

The Examiner asserted that the issue to be determined is whether the invention as claimed could be selected from Szabo et al. which taught a list of known multiple sclerosis-agents that 'could' be selected for combinatorial therapy for multiple sclerosis. The Examiner then asserted that a list of 5 agents, and the express teaching (or at a minimum) that any of the 5 therein may be used concurrently in a combination therapy is simply too overwhelming evidence that such would have been predictable, in the form applicant has presently claimed based on the predictable results that both agents presently claimed were known for use as multiple sclerosis-treatment agents.

Applicants' Response

In response, applicants respectfully traverse the obviousness rejection for each of the following reasons independently.

I. Applicants' Claimed Method and Result are Unpredictable

Based on clinical experimentation, applicants have made the unexpected observation that immunosuppression with mitoxantrone accelerates and enhances the efficacy of glatiramer acetate administered to the patient (see, e.g. page 15, lines 15-18 of the subject application; and abstract of Vollmer et al., *Multiple Sclerosis* 2008; 14: 663-670). Specifically,

- applicants observed that mitoxantrone-glatiramer acetate induction produced an 89% greater reduction in the number of Gd-enhancing lesions at months 6 and 9 and 70% reduction at months 12 and 15 versus glatiramer acetate alone;
- mean relapse rates were 0.16 and 0.32 in the mitoxantrone-glatiramer acetate and glatiramer acetate groups, respectively; and
- short-term immunosuppression with mitoxantrone followed by daily GA for up to 15 months was found to be safe and

effective, with an early and sustained decrease in MRI disease activity.

II. Prior Art Shows that Applicants' Claimed Method is Unpredictable

It is well established in the prior art that individual drugs known to treat multiple sclerosis, are not necessarily compatible for a combination therapy. Applicants' own publication (Vollmer et al.) identifies at least one such case as cited in the Amendment in Response to April 3, 2009 Office Action dated August 3, 2009. Specifically, Vollmer et al. recites that "certain combinations, such as natalizumab [an immunoglobulin] and interferon β-1α [an interferon] may also increase the risk of unanticipated side effects." Importantly, both "interferon" and "immunoglobulin" are two of the five agents recited in claim 11 of Szabo et al., upon which the Examiner relies. The teaching of Vollmer et al. is further supported by Rudick et al. *New England Journal of Medicine* 2006; 354: 911-923; B.K. Kleinschmidt-DeMasters, M.D., and Kenneth L. Tyler, M.D. *New England Journal of Medicine* 2005; 353: 369-379; and Langer-Gould et al. *New England Journal of Medicine* 2005; 353: 369-379 which all describe the association of serious adverse effects with the drug combination of natalizumab and interferon β-1α (attached hereto as **Exhibits 1-3** respectively).

There are yet further examples rebutting the Examiner's assertion of predictability. Specifically:

- Brod et al. (Annals of Neurology, 2000 (47); 127-131, attached hereto as **Exhibit 4**) provide experimental data in the EAE disease model comparing each of glatiramer acetate and interferon administration to a combined administration of glatiramer acetate and interferon. The experiments clearly demonstrate that "a combination of

either ingested or subcutaneous murine IFN-α in conjunction with glatiramer acetate abrogates the clinical effectiveness of either therapy." (See Brod et al., page 130, top of 2nd column.) Again, importantly, both "glatiramer acetate" and "interferon" are two of the five agents recited in claim 11 of Szabo et al., upon which the Examiner relies. Brod et al. further proposes that the administration of interferon "could result in ablation of the therapeutic efficacy of glatiramer acetate by inhibition of glatiramer acetate Th2 T-cell lymphocyte infiltration into the CNS" and that the action of glatiramer acetate may "inhibit the activity of type I interferon by stimulating the production of IL-10 and IL-4 from responsive Th2 cells" which results in "the benefits of either drug used alone are lost." (See Brod et al., page 130, 2nd column, 3rd full paragraph.)

- Salama et al. (Multiple Sclerosis, 2003 (9); 28-31, attached hereto as **Exhibit 5**) examines whether the combination of beta-IFN (an interferon) with prednisone (a glucocorticoid) would enhance the immunoregulatory effects of beta-IFN in clinically definite multiple sclerosis patients by measuring serum levels of selected proinflammatory cytokines and soluble T-cell activation markers associated with multiple sclerosis. (See abstract and page 28, 1st column.) Salama et al. shows that compared to patients treated with beta-IFN alone, "the addition of prednisone at the given dose in the combination therapy appeared to antagonize the up-regulatory effect of beta-IFN-1a..." (emphasis added)(See page 29, paragraph spanning the 1st and 2nd column.) Again, "a glucocorticoid" and "an interferon" are two of the five agents recited in claim 11 of Szabo et al., upon which the Examiner relies.

III. Examiner's Predictability Assumption is Contrary to Views of Individuals of Ordinary Skill in This Art

Finally, it is well known that the administration of two different drugs to treat a given condition, such as a form of multiple sclerosis, raises a number of potential problems (see pages 4, line 26 to page 5, line 29 of the subject application). Specifically, as taught by the United States Food and Drug Administration, in vivo drug interactions are complex and when two drugs are administered to treat the same condition, it is unpredictable whether the many metabolic routes of the human body will "be inhibited, activated, or induced by concomitant drug treatment" thus making it unpredictable whether each drug will complement, have no effect on, or interfere with the therapeutic activity of the other in a human subject. (See Guidance for Industry-*In vivo* drug metabolism/drug interaction studies-study design, data analysis and recommendations for dosing and labeling, page 2, beginning of section B, attached hereto as **Exhibit 6**.) In addition, the interaction between two drugs "can be substantial… and can include formation of toxic metabolites or increased exposure to a toxic parent compound." (See Guidance for Industry-*In vivo* drug metabolism/drug interaction studies-study design, data analysis and recommendations for dosing and labeling, bottom of page 2, section B.); "Even drugs that are not substantially metabolized can have important effects on the metabolism of concomitant drugs." (See Guidance for Industry-*In vivo* drug metabolism/drug interaction studies-study design, data analysis and recommendations for dosing and labeling, page 3, 3rd bullet point.) Moreover, it is difficult to accurately predict when the effects of the interaction between two drugs will be manifest;

> *Unlike relatively fixed influences on metabolism, such as hepatic function or genetic characteristics, metabolic*

> *drug-drug interactions can lead to abrupt changes in exposure. Depending on the nature of the drugs, these effects could potentially occur when a drug is initially administered, when it has been titrated to a stable dose, or when an interacting drug is discontinued. Interactions can occur after even a single concomitant dose of an inhibitor.* (See Guidance for Industry-*In vivo* drug metabolism/drug interaction studies study design, data analysis and recommendations for dosing and labeling, page 4, 4th bullet point.)

Accordingly, the prior art does not reasonably predict success when administering two drugs for a given disease as asserted by the Examiner and certainly not for multiple sclerosis as evidenced by multiple reports of failure. In fact, the prior art provides no evidence or even suggestion that treatment with mitoxantrone would enhance the efficacy of glatiramer acetate in general or by reducing the accumulation of Gd-enhancing lesions or mean relapse rate.

Applicants also note that predictability must be analyzed taking into account views of those skilled in the art at the time the invention was made. The views of the FDA are clearly relevant and show known obstacles to predicting the effects of administration of a combination of two drugs to treat a given condition (see Guidance for Industry-*In vivo* drug metabolism/drug interaction studies-study design, data analysis and recommendations for dosing and labeling). Dr. Vollmer has shown that the claimed combination is effective and not subject to unexpected side effects, which is unlike prior art combination treatment attempts e.g. glatiramer acetate and interferon, beta-IFN and prednisone, or natalizumab and interferon β-1α discussed herein above. The results and skepticism of Vollmer et al. have been published in a peer-reviewed international journal i.e., *Multiple Sclerosis*. Accordingly, contrary to the Examiner's

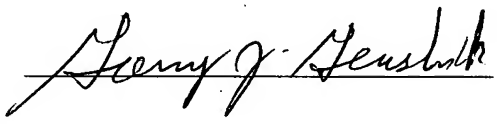
assertion, the state of the art and the opinion of skilled artisans show that applicants' claimed invention is not predictable.

In conclusion, applicant submits that Szabo et al. do not disclose the co-administration of mitoxantrone and glatiramer acetate for the treatment of multiple sclerosis as recited in the pending claims. Moreover, neither Arnon et al. nor Kerwar et al. provide any motivation or guidance to predictably overcome the known obstacles to predicting the effects of a combination of two drugs to treat a given condition. Thus, none of the Examiner's cited references, alone or in combination, support the obviousness rejection.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone him at the number provided below.

No fee is deemed necessary in connection with the filing of this response. However, if any fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,



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Gary J. Gershik Date
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Exhibit 1

ORIGINAL ARTICLE

Natalizumab plus Interferon Beta-1a for Relapsing Multiple Sclerosis

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ABSTRACT

BACKGROUND

Interferon beta is used to modify the course of relapsing multiple sclerosis. Despite interferon beta therapy, many patients have relapses. Natalizumab, an α_4 integrin antagonist, appeared to be safe and effective alone and when added to interferon beta-1a in preliminary studies.

METHODS

We randomly assigned 1171 patients who, despite interferon beta-1a therapy, had had at least one relapse during the 12-month period before randomization to receive continued interferon beta-1a in combination with 300 mg of natalizumab (589 patients) or placebo (582 patients) intravenously every 4 weeks for up to 116 weeks. The primary end points were the rate of clinical relapse at 1 year and the cumulative probability of disability progression sustained for 12 weeks, as measured by the Expanded Disability Status Scale, at 2 years.

RESULTS

Combination therapy resulted in a 24 percent reduction in the relative risk of sustained disability progression (hazard ratio, 0.76; 95 percent confidence interval, 0.61 to 0.96; P=0.02). Kaplan–Meier estimates of the cumulative probability of progression at two years were 23 percent with combination therapy and 29 percent with interferon beta-1a alone. Combination therapy was associated with a lower annualized rate of relapse over a two-year period than was interferon beta-1a alone (0.34 vs. 0.75, P<0.001) and with fewer new or enlarging lesions on T_2-weighted magnetic resonance imaging (0.9 vs. 5.4, P<0.001). Adverse events associated with combination therapy were anxiety, pharyngitis, sinus congestion, and peripheral edema. Two cases of progressive multifocal leukoencephalopathy, one of which was fatal, were diagnosed in natalizumab-treated patients.

CONCLUSIONS

Natalizumab added to interferon beta-1a was significantly more effective than interferon beta-1a alone in patients with relapsing multiple sclerosis. Additional research is needed to elucidate the benefits and risks of this combination treatment. (ClinicalTrials.gov number, NCT00030966.)

From the Mellen Center for Multiple Sclerosis Treatment and Research, Cleveland Clinic Foundation, Cleveland (R.A.R.); the MS Center of Atlanta, Atlanta (W.H.S.); the Johns Hopkins Multiple Sclerosis Center, Baltimore (P.A.C.); Hôpital Neurologique, Lyon, France (C.C.); University of Pennsylvania School of Medicine, Philadelphia (S.L.G.); University Hospital Basel, Basel, Switzerland (E.-W.R.); Mt. Sinai School of Medicine, New York (F.D.L.); Baird Multiple Sclerosis Center, State University of New York at Buffalo, Buffalo (B.W.-G.); Consultants in Neurology Multiple Sclerosis Center, Northbrook, Ill. (D.R.W.); and Biogen Idec, Cambridge, Mass. (F.L., M.A.P., A.W.S.). Address reprint requests to Dr. Rudick at the Mellen Center for Multiple Sclerosis Treatment and Research, Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195, or at rudickr@ccf.org.

*The Safety and Efficacy of Natalizumab in Combination with Interferon Beta-1a in Patients with Relapsing Remitting Multiple Sclerosis (SENTINEL) Investigators are listed in the Supplementary Appendix, available with the full text of this article at www.nejm.org.

N Engl J Med 2006;354:911-23.

THE ADHESION MOLECULE $\alpha_4\beta_1$ INTEGRIN is a key initiator of the inflammatory cascade involved in the pathogenesis of multiple sclerosis.[1-4] Natalizumab (Tysabri, Biogen Idec and Elan Pharmaceuticals) is the first α_4 integrin antagonist in a new class of selective adhesion-molecule inhibitors for the treatment of multiple sclerosis. Natalizumab binds to α_4 integrin on the surface of leukocytes, inhibiting their migration into the brain and thereby reducing inflammation.

Current disease-modifying therapies for relapsing–remitting multiple sclerosis (interferon beta and glatiramer acetate) are only partially effective,[5-8] and most patients with multiple sclerosis have breakthrough disease activity despite therapy with these agents. Hence, there is a need for additional treatment options in multiple sclerosis. Natalizumab is an attractive therapy to add to current disease-modifying therapies in patients with breakthrough disease because preliminary efficacy[9] and safety[10] data have been favorable and because the mechanism of action of natalizumab may complement those of other disease-modifying therapies.[11-17]

The Safety and Efficacy of Natalizumab in Combination with Interferon Beta-1a in Patients with Relapsing Remitting Multiple Sclerosis (SENTINEL) study was a two-year, phase 3 clinical trial designed to determine whether natalizumab, when added to interferon beta-1a, has efficacy in addition to that associated with interferon beta-1a alone. The trial was also designed to confirm the safety of natalizumab when added to interferon beta-1a.

METHODS

PATIENTS

One hundred twenty-four clinical centers in Europe and the United States enrolled 1196 patients beginning on January 14, 2002. All patients gave written informed consent. The study protocol was developed by the investigator advisory committee and the sponsors and was approved by central and local ethics committees, and the study was overseen by an independent safety-monitoring committee. Data were collected by the investigators and an independent organization (PPD International) and were held and analyzed by Biogen Idec and Elan Pharmaceuticals. During the study, the investigator advisory committee and representatives of Biogen Idec met at least monthly to review and manage the study. The manuscript was written by Drs. Rudick and Panzara, with input from each of the other authors; all the authors vouch for the veracity and completeness of the data and analyses.

Eligible patients were 18 to 55 years of age; had a diagnosis of relapsing–remitting multiple sclerosis,[18] a score on the Expanded Disability Status Scale (EDSS) (possible scores range from 0 to 10, with higher scores indicating more severe disease) between 0 and 5.0,[19] and a magnetic resonance imaging (MRI) scan revealing lesions consistent with a diagnosis of multiple sclerosis; had received treatment with interferon beta-1a for at least 12 months before randomization; and had had at least one relapse during the 12-month period before randomization. Patients were ineligible if they had primary progressive, secondary progressive, or progressive relapsing multiple sclerosis[20]; if they had had a relapse within 50 days before randomization; or if they had been treated with an approved disease-modifying therapy other than interferon beta-1a intramuscularly once weekly within the 12-month period before randomization.

STUDY DESIGN AND RANDOMIZATION

This study was a randomized, double-blind, placebo-controlled, parallel-group, phase 3 clinical trial. Data from 1171 of the 1196 patients enrolled were analyzed, because a single center with 25 patients was excluded before unblinding owing to irregularities in data. Patients were randomly assigned, in a 1:1 ratio, to receive 300 mg of natalizumab (589 patients) or placebo (582 patients) intravenously every 4 weeks in addition to interferon beta-1a (Avonex, Biogen Idec) at a dose of 30 μg intramuscularly once weekly for up to 116 weeks. Randomization was stratified according to study site in blocks of four (two active and two placebo) with the use of a computer-generated schedule and a multidigit identification number, implemented by way of an interactive voice-response system. All study personnel, patients, sponsor personnel involved in the conduct of the study, and members of the investigator advisory committee were blinded to the treatment assignments throughout the study.

STUDY PROCEDURE AND END POINTS

Each site designated primary and backup examining neurologists and treating neurologists. The

examining neurologists performed the EDSS and neurologic examinations but were otherwise not involved in the patients' medical care. The treating neurologists were responsible for all patient care, including the management of adverse events and relapses of multiple sclerosis.

Clinical visits every 12 weeks included assessment of relapses, EDSS evaluation, blood chemical and hematologic tests, assessment of any adverse events, and immunogenicity studies. Patients were also seen by a treating neurologist during unscheduled visits within 72 hours after the development of new symptoms so that they could be assessed for possible relapses or adverse events. If a relapse was suspected, the patient was evaluated by the examining neurologist. Relapses were defined as the development of new or recurrent neurologic symptoms not associated with fever or infection, lasting at least 24 hours, and accompanied by new, objective neurologic findings. At the discretion of the treating neurologist, relapses were treated with intravenous methylprednisolone at a dose of 1000 mg per day for three or five days. Patients who had disability progression that was sustained for 12 weeks were asked to provide consent to continue study participation and were given the option of adding an available multiple sclerosis treatment as rescue medication, according to protocol, while continuing to receive the study drug. Patients who discontinued the study drug were strongly encouraged to remain in the study for follow-up assessments, and all patients who continued to participate in the study were evaluated (according to the intention-to-treat principle).

Proton-density, T_2-weighted MRI scans and gadolinium-enhanced T_1-weighted MRI scans of the brain were obtained at baseline and at weeks 52 and 104. Forty contiguous, 3-mm-thick axial slices were acquired. MRI analyses were performed centrally at the MS-MRI Evaluation Center (Basel, Switzerland) by blinded raters. The scans were checked for artifacts, compliance with scanning requirements, and repositioning.

The primary efficacy end point was the rate of clinical relapse at one year. Secondary end points at one year were the number of new or enlarging T_2-hyperintense lesions, the number of gadolinium-enhancing lesions, and the proportion of patients free of relapse. The primary efficacy end point at two years was the cumulative probability of sustained disability progression, defined as an increase by at least 1.0 point in the EDSS score from a baseline score of at least 1.0 or an increase by at least 1.5 points in the EDSS score from a baseline score of 0, sustained for 12 weeks; progression could not be confirmed during a relapse. Secondary end points at two years were the rate of clinical relapse, the volume of T_2-hyperintense lesions, the number of new T_1-hypointense lesions, and disability as measured by the Multiple Sclerosis Functional Composite.[21] This report presents data pertaining to primary end points and key secondary efficacy end points, as well as safety data. Results pertaining to additional secondary end points and tertiary end points are not included in this report.

Binding antibodies against natalizumab were assessed with use of an enzyme-linked immunosorbent assay. Positive samples (0.5 μg per milliliter) were further tested in a flow-cytometry assay to determine whether these antibodies interfered with the binding of natalizumab to α_4 integrin.

STATISTICAL ANALYSIS

The sample size was estimated, on the basis of data from previous trials of natalizumab[9] and interferon beta-1a,[6] with the use of two-sided tests with an experiment-wise alpha of 0.05. The annualized rate of relapse among patients receiving combination therapy at one year was predicted to be 0.6, as compared with 0.9 among patients receiving interferon beta-1a alone. For the annualized relapse rate, the likelihood-ratio test was used to determine the sample size with half the patients receiving active drug and half receiving placebo. With an assumed dropout rate of 17 percent, rounding, a type I error rate of 2.5 percent, and a type II error rate of 90 percent, the number of patients needed was estimated to be 1200. To power the study for the two-year end point of disability progression, we assumed a progression rate of 34.9 percent at the end of two years in the group assigned to interferon beta-1a alone and a progression rate of 22.7 percent at the end of two years (a 35 percent improvement) in the combination-therapy group. Simulations of the log-rank test were run with 60 percent of the accrual in the first 24 weeks and the remainder in the next 24 weeks. With an assumed dropout rate of 20 percent, the sample size of 1200 provided at least 92 percent power with a Bonferroni adjustment for multiple end points and with the type I error rate maintained at 5 percent.

The baseline characteristics of the patients were analyzed with the use of a t-test, with the exceptions of sex, race, and diagnosis of multiple sclerosis (based on the McDonald criteria[18]), which were analyzed with the use of a chi-square test. The time to the onset of disability progression sustained for 12 weeks was used to determine the cumulative probability of disability progression estimated by the Kaplan–Meier method. The Cox proportional-hazards model, adjusted for the baseline EDSS score, was used to compare the Kaplan–Meier curves. The annualized relapse rate was calculated by Poisson regression and adjusted for the number of relapses in the year before randomization; data pertaining to relapses that occurred after rescue treatment was initiated (per protocol) were censored. Additional baseline factors were tested for inclusion in each of the models: EDSS score (≤3.5 or >3.5), gadolinium-enhancing lesions (present or absent), the number of T_2-hyperintense lesions (<9 or ≥9), and age (<40 or ≥40 years).[22-24] Each covariate was tested in the model for statistical significance by a backward-selection procedure, and only statistically significant covariates ($P \leq 0.10$) were included in the final models. No additional covariates were included in the analysis of disability progression. Three additional covariates (baseline EDSS score, the presence or absence of gadolinium-enhancing lesions at baseline, and age) were included in the analysis of relapse rate.

A sensitivity analysis of disability progression (based on the change in EDSS score) sustained for 24 weeks was also conducted. For the annualized rate of relapse, sensitivity analyses were performed with and without censoring, as well as with and without adjustment for significant covariates. The unadjusted rate of relapse was calculated as the total number of relapses divided by the total number of subject-years of follow-up in each treatment group. The Hochberg procedure[25] for multiple comparisons was used in the analysis of the two primary end points; hence, the significance level was set such that if the higher of the P values for the analyses of these end points was less than or equal to 0.05, then both end points were considered to be statistically significant; otherwise, the lower of the P values was tested at a significance level of 0.025.

Secondary efficacy end points were ranked ordered, and a closed testing procedure was used such that if statistical significance was not achieved for a given end point, then end points of a lower rank were considered not statistically significant. Secondary efficacy end points were analyzed by logistic regression with a term for treatment group and with their respective baseline values as covariates; missing values were imputed by using the mean in the study population.

Adverse events were analyzed with use of the chi-square test, and serious adverse events were analyzed with use of Fisher's exact test. Poisson regression was used to calculate the difference between the rates of infection in each treatment group.

All analyses followed the intention-to-treat principle. All reported P values are two-tailed. The date on which the database was locked for the two-year analyses was May 31, 2005, and as a result there were 2528 patient-years of observation and 1222 patient-years of exposure to natalizumab.

RESULTS

PATIENTS

SENTINEL was stopped approximately one month early, on February 28, 2005, because of two reports of progressive multifocal leukoencephalopathy (PML). Of the 1171 patients, a total of 1003 (86 percent) completed the 120-week study; 168 patients (14 percent overall; 12 percent of the group assigned to interferon beta-1a plus natalizumab and 16 percent of the group assigned to interferon beta-1a alone) withdrew from the study (Fig. 1). Sixty-four patients discontinued the study drug but completed follow-up (5 percent overall; 5 percent of the combination-therapy group and 6 percent of the group assigned to interferon beta-1a alone). There were no significant differences in demographic or disease-related characteristics at baseline between the two treatment groups, with the exception of the duration of disease (median, seven years in the combination-therapy group and eight years in the group assigned to interferon beta-1a alone; P=0.02) (Table 1).

The SENTINEL data represent 28 percent of the placebo-controlled experience with natalizumab (in terms of patient-years of exposure) in both multiple sclerosis and Crohn's disease and 44 percent of the overall experience in multiple sclerosis.

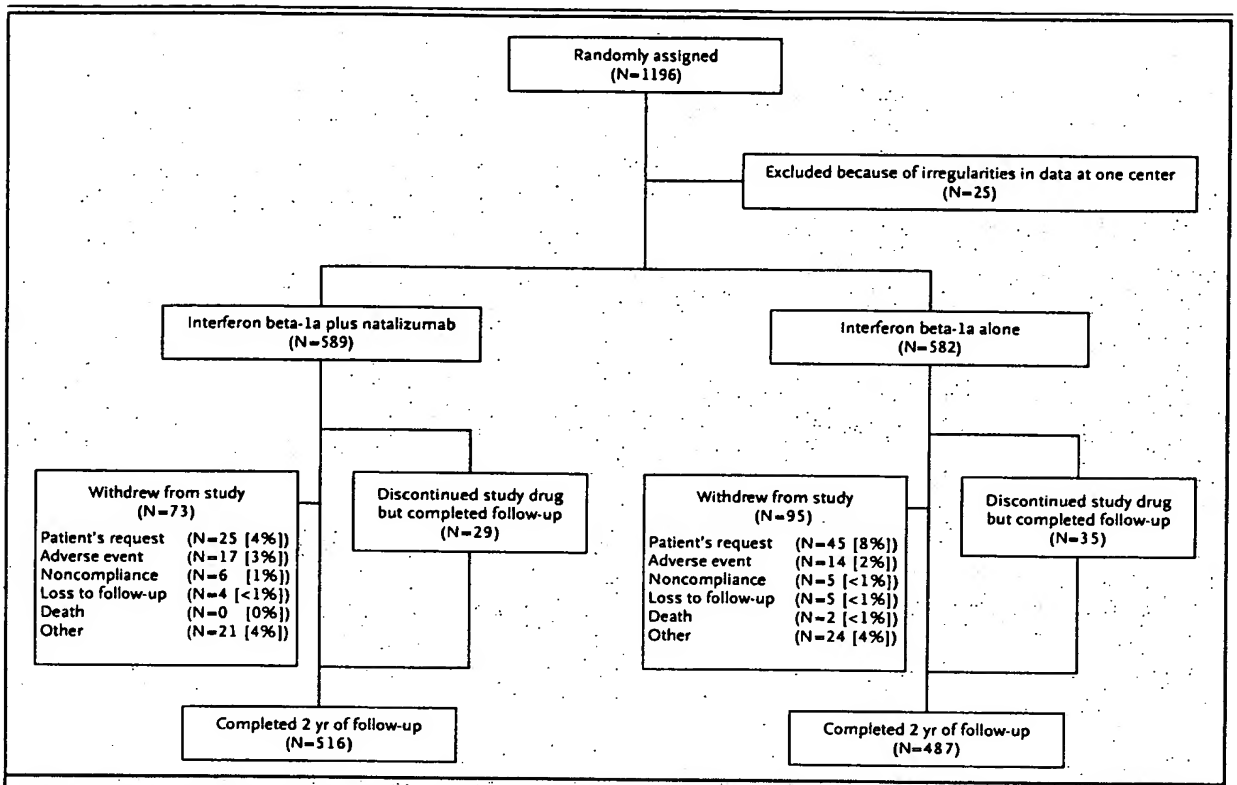

Figure 1. Disposition of Patients during the Two-Year SENTINEL Study.

EFFICACY

Kaplan–Meier estimates of the cumulative probability of sustained disability progression at 2 years were 23 percent with combination therapy and 29 percent with interferon beta-1a alone (Fig. 2 and Table 2). Combination therapy resulted in a 24 percent decrease in the risk of sustained disability progression (hazard ratio, 0.76; 95 percent confidence interval, 0.61 to 0.96; P=0.02). In the sensitivity analysis of the risk of disability progression sustained for 24 weeks, estimates of the cumulative probability of progression by 2 years were 15 percent for combination therapy and 18 percent for interferon beta-1a alone (representing an 18 percent reduction with combination therapy); however, this difference was not statistically significant (P=0.17).

Combination therapy reduced the annualized rate of relapse at one year, which was 0.82 with interferon beta-1a alone, to 0.38 (P<0.001) — a 54 percent reduction (Table 2). This difference was maintained at two years, at which time the rate was 0.75 with interferon beta-1a alone and 0.34 with combination therapy (a 55 percent reduction with combination therapy, P<0.001). Subgroup analyses (according to relapse history, EDSS score, age, sex, the presence or absence of gadolinium-enhancing lesions, and the number of T_2-hyperintense lesions) and a sensitivity analysis of relapse rate showed consistent results. The proportion of patients who were relapse-free at two years was 54 percent in the combination-therapy group, as compared with 32 percent in the group assigned to interferon beta-1a alone (P<0.001). The risk of relapse was 50 percent lower with combination therapy (hazard ratio, 0.50; 95 percent confidence interval, 0.43 to 0.59; P<0.001).

The number of new or enlarging T_2-hyperintense lesions over the two-year period was re-

Table 1. Baseline Characteristics of the Patients.*

Characteristic	Interferon Beta-1a plus Natalizumab (N=589)	Interferon Beta-1a Alone (N=582)	Total (N=1171)
Age — yr			
Mean ±SD	38.8±7.7	39.1±7.6	38.9±7.7
Range	18–55	19–55	18–55
Sex — no. of patients (%)			
Male	147 (25)	162 (28)	309 (26)
Female	442 (75)	420 (72)	862 (74)
Race — no. of patients (%)†			
White	550 (93)	542 (93)	1092 (93)
Other	39 (7)	40 (7)	79 (7)
Duration of disease — yr			
Median	7.0‡	8.0	7.0
Range	1–34	1–34	1–34
No. of relapses in previous 1 yr — no. of patients (%)			
0	0	1 (<1)	1 (<1)
1	390 (66)	357 (61)	747 (64)
2	153 (26)	174 (30)	327 (28)
≥3	44 (7)	50 (9)	94 (8)
Missing data	2 (<1)	0	2 (<1)
No. of relapses in previous 1 yr			
Mean ±SD	1.44±0.75	1.49±0.72	1.47±0.73
Range	1–7	0–5	0–7
EDSS score — no. of patients (%)§			
0	24 (4)	19 (3)	43 (4)
1.0–1.5	145 (25)	143 (25)	288 (25)
2.0–2.5	214 (36)	203 (35)	417 (36)
3.0–3.5	125 (21)	126 (22)	251 (21)
4.0–4.5	68 (12)	72 (12)	140 (12)
5.0	12 (2)	16 (3)	28 (2)
≥5.5	1 (<1)	3 (<1)	4 (<1)
EDSS score			
Mean ±SD	2.4±1.1	2.5±1.1	2.4±1.1
Range	0–6.0	0–5.5	0–6.0

duced from 5.4 with interferon beta-1a alone to 0.9 with combination therapy (P<0.001), representing an 83 percent reduction with combination therapy (Table 2). The mean number of gadolinium-enhancing lesions at two years was 0.9 with interferon beta-1a alone and 0.1 with combination therapy, representing an 89 percent reduction (P<0.001).

SAFETY

At least one adverse event was reported by 584 patients assigned to receive interferon beta-1a plus natalizumab (>99 percent) and 578 assigned to receive interferon beta-1a alone (>99 percent). Adverse events significantly associated with combination therapy were anxiety, pharyngitis, sinus congestion, and peripheral edema (Table 3). The

Table 1. (Continued.)

Characteristic	Interferon Beta-1a plus Natalizumab (N=589)	Interferon Beta-1a Alone (N=582)	Total (N=1171)
No. of gadolinium-enhancing lesions — no. of patients (%)			
0	392 (67)	374 (64)	766 (65)
1	98 (17)	105 (18)	203 (17)
2	31 (5)	32 (5)	63 (5)
3	20 (3)	26 (4)	46 (4)
≥4	43 (7)	42 (7)	85 (7)
Missing data	5 (<1)	3 (<1)	8 (<1)
No. of gadolinium-enhancing lesions			
Mean ±SD	0.9±2.5	0.9±1.9	0.9±2.2
Range	0–24	0–16	0–24
No. of T_2-hyperintense lesions — no. (%)			
<9	67 (11)	52 (9)	119 (10)
≥9	519 (88)	528 (91)	1047 (89)
Missing data	3 (<1)	2 (<1)	5 (<1)
Duration of interferon beta-1a therapy before study — mo			
Mean ±SE	33.6±0.7	35.4±0.7	34.5±0.5
Median	29.0	32.0	31.0
Range	10–88	11–99	10–99

* Percentages may not total 100, because of rounding.
† Race was determined at the time of enrollment by the treating investigator.
‡ P≤0.02 for the comparison with the group assigned to interferon beta-1a alone.
§ EDSS denotes the Expanded Disability Status Scale (possible scores range from 0 to 10, with higher scores indicating more severe disease).

worst adverse events associated with combination therapy were mild in 10 percent of the patients, moderate in 54 percent, and severe in 35 percent; the respective percentages for interferon beta-1a alone were 5 percent, 57 percent, and 37 percent. Serious adverse events were observed in 18 percent of the patients assigned to combination therapy and 21 percent of those assigned to interferon beta-1a alone (P=0.23). The most common serious adverse event was a relapse of multiple sclerosis, which occurred in 5 percent of the patients in the combination-therapy group and 9 percent of those in the interferon beta-1a group (P=0.002). One of the serious adverse events reported was PML, which occurred in a patient who had received 29 doses of natalizumab. A second patient received a diagnosis of PML after her completion of the two-year study and after she had received 37 doses of natalizumab. The details of these cases of PML have been reported previously.[26,27] Two of the patients assigned to interferon beta-1a alone died: one was a 47-year-old woman with a history of sinus arrhythmia and heart murmur, and the other was a 23-year-old woman with a history of headache, pain, and use of prescribed methadone who died during sleep.

Depression was assessed every six months with use of the Beck Depression Inventory II.[28] There were no differences between the treatment groups in Beck Depression Inventory II scores during the study (data not shown).

The incidence of infection was 83 percent in the combination-therapy group and 81 percent in the group assigned to interferon beta-1a alone; infections occurred at a rate of 1 per patient-year in each group. When the data pertaining to infection were reanalyzed to include multiple occurrences, the rate increased in each group, as expected. However, there remained no significant difference between the groups, with infection rates of 1.54 per patient-year with combination therapy and 1.53 per patient-year with interferon

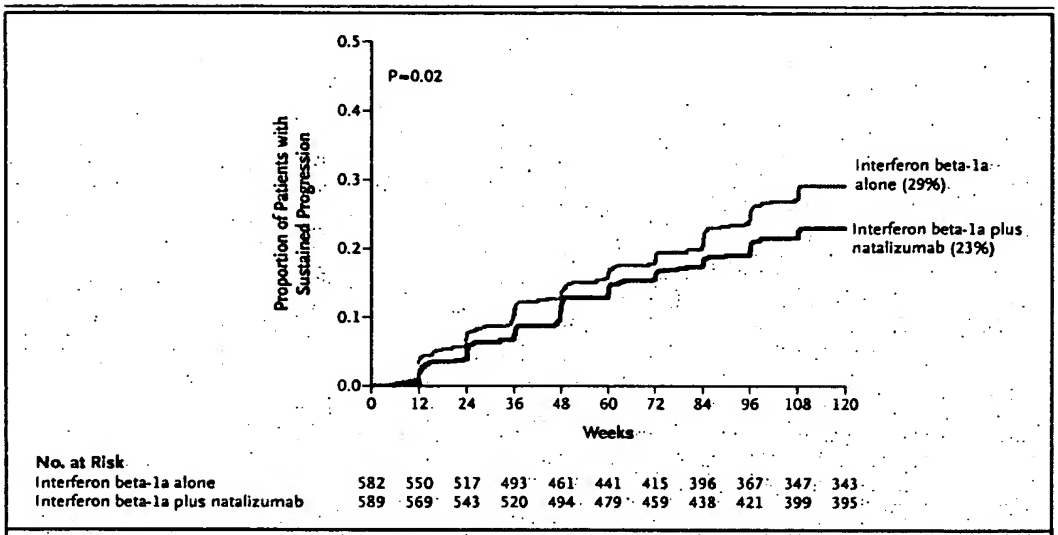

Figure 2. Kaplan–Meier Plots of the Time to Sustained Disability Progression.

The hazard ratio for sustained progression in the combination-therapy group as compared with the group given interferon beta-1a alone was 0.76 (95 percent confidence interval, 0.61 to 0.96).

beta-1a alone (P=0.95). Common infections were nasopharyngitis (39 percent vs. 35 percent); urinary tract infection, not otherwise specified (18 percent vs. 19 percent); sinusitis, not otherwise specified (18 percent vs. 15 percent); upper respiratory tract infection, not otherwise specified (17 percent vs. 18 percent); and influenza (17 percent vs. 15 percent). Serious infections occurred in 2.7 percent and 2.9 percent of the patients assigned to combination therapy and interferon beta-1a alone, respectively. There were no cases of tuberculosis. The incidence of cancer was 1 percent in the combination-therapy group and 2 percent in the group assigned to interferon beta-1a alone.

Infusion reactions, defined as any event occurring within two hours after the start of an infusion, occurred in 24 percent of the patients in the combination-therapy group and 20 percent of those in the group assigned to interferon beta-1a alone (P=0.11). The most common infusion reaction was headache. Most reactions were treated symptomatically and did not result in discontinuation of the study drug. Hypersensitivity reactions included all events reported on the basis of clinical judgment as hypersensitivity, an allergic reaction, an anaphylactic or anaphylactoid reaction, urticaria, or hives by the investigator and were categorized according to severity. Eleven patients assigned to combination therapy (1.9 percent) had a hypersensitivity reaction; 8 of the 11 hypersensitivity reactions were isolated cases of urticaria (2 of which were severe). In addition, two patients assigned to interferon beta-1a alone (0.3 percent) had hypersensitivity reactions associated with the infusion of placebo; both were cases of mild urticaria. There was no cardiopulmonary compromise associated with any event. Natalizumab was discontinued, and the episodes resolved without sequelae.

Eight percent of the patients in the combination-therapy group and 7 percent of those in the group assigned to interferon beta-1a alone discontinued the study drug because of an adverse event. Three percent and 2 percent, respectively, withdrew from the study because of an adverse event.

Natalizumab-treated patients had increases in lymphocytes, monocytes, eosinophils, and basophils — changes consistent with the drug's known pharmacodynamic effects and the presence of α_4 integrins on these cell types. Increases in nucleated red cells also were seen transiently in a small number of patients. These laboratory changes were not associated with any clinical manifestations and were reversible, with values returning to baseline within 16 weeks after the last dose. Elevations in neutrophils were not observed. No

Table 2. Clinical and Magnetic Resonance Imaging End Points.*

End Point	1 Yr			2 Yr		
	Interferon Beta-1a plus Natalizumab (N=589)	Interferon Beta-1a Alone (N=582)	P Value	Interferon Beta-1a plus Natalizumab (N=589)	Interferon Beta-1a Alone (N=582)	P Value
Clinical						
Primary end point at 2 yr: cumulative probability of sustained disability progression — %†	—	—		23	29	0.02‡
Primary end point at 1 yr: annualized relapse rate — mean (95% CI)§						
Preplanned interim analysis (after 1200 patient-yr)	0.38 (0.33–0.45)	0.82 (0.72–0.92)	<0.001			
Final analysis	0.38 (0.32–0.45)	0.81 (0.72–0.92)	<0.001	0.34 (0.29–0.39)	0.75 (0.67–0.84)	0.001
No. of relapses — no. of patients (%)						
0	424 (72)	296 (51)		359 (61)	217 (37)	
1	132 (22)	183 (31)		158 (27)	164 (28)	
2	30 (5)	77 (13)		41 (7)	105 (18)	
≥3	3 (<1)	26 (4)		31 (5)	96 (16)	
Sensitivity analysis¶						
Adjusted annualized rate of relapse	—	—		0.34 (0.29–0.39)	0.75 (0.67–0.84)	0.001
Unadjusted annualized rate of relapse	—	—		0.31	0.70	0.001
Per-subject mean relapse rate‖	—	—		0.33	0.75	0.001
Magnetic resonance imaging						
No. of new or enlarging T_2-hyperintense lesions — no. of patients (%)			<0.001			0.001
0	422 (72)	248 (43)		394 (67)	176 (30)	
1	108 (18)	114 (20)		76 (13)	55 (9)	
2	32 (5)	66 (11)		39 (7)	59 (10)	
≥3	27 (5)	154 (26)		80 (14)	292 (50)	
No. of new or enlarging T_2-hyperintense lesions						
Mean ±SD	0.5±1.2	2.4±4.1		0.9±2.1	5.4±8.7	
Median	0	1.0		0	3	
Minimum, maximum	0, 14	0, 28		0, 27	0, 64	
No. of gadolinium-enhancing lesions — no. of patients (%)			<0.001			0.001
0	563 (96)	436 (75)		568 (96)	435 (75)	
1	19 (3)	73 (13)		13 (2)	67 (12)	
2	3 (<1)	28 (5)		4 (<1)	33 (6)	
≥3	4 (<1)	45 (8)		4 (<1)	47 (8)	
No. of gadolinium-enhancing lesions						
Mean ±SD	0.1±0.4	0.8±2.5		0.1±0.6	0.9±3.2	
Median	0	0		0	0	
Minimum, maximum	0, 4	0, 43		0, 12	0, 43	

* P values are for the comparison between the combination-therapy group and the group assigned to interferon beta-1a alone. CI denotes confidence interval.
† Sustained disability progression was defined as an increase by at least 1.0 point in the Expanded Disability Status Scale (EDSS) score from a baseline score of at least 1.0 or an increase by at least 1.5 points in the EDSS score from a baseline score of 0, sustained for 12 weeks. Progression could not be confirmed during a relapse.
‡ The hazard ratio for sustained disability progression in the combination-therapy group as compared with the group assigned to interferon beta-1a alone was 0.76 (95 percent confidence interval, 0.61 to 0.96).
§ Data pertaining to relapses that occurred after sustained progression was reached and rescue treatment was initiated (per protocol) were censored.
¶ The sensitivity analysis included relapses that occurred after sustained progression was reached and rescue treatment was initiated (per protocol).
‖ The per-subject mean relapse rate is the number of relapses for each patient divided by the total number of years of follow-up in the study for that patient.

Table 3. Adverse Events.*

Event	Interferon Beta-1a plus Natalizumab (N=589)	Interferon Beta-1a Alone (N=582)
	percent of patients	
Adverse events†		
Headache	46	44
Nasopharyngitis	39	35
Pain in arms or legs	22	21
Depression	21	18
Influenza-like illness	20	19
Diarrhea, NOS	19	16
Insomnia	18	17
Sinusitis, NOS	18	15
Influenza	17	15
Nausea	17	15
Myalgia	13	10
Anxiety	12‡	8
Cough	11	9
Viral upper respiratory tract infection, NOS	8	7
Pharyngitis	7§	4
Vomiting, NOS	7	5
Muscle cramp	6	5
Abdominal pain, NOS	6	5
Sinus congestion	6‡	3
Seasonal allergy	6	4
Peripheral edema	5¶	1
Tremor	5	3
Sinus headache	5	3

Event	Interferon Beta-1a plus Natalizumab (N=589)	Interferon Beta-1a Alone (N=582)
	percent of patients	
Serious adverse events‖		
Relapse of multiple sclerosis	5‡	9
Appendicitis	<1	<1
Abdominal pain, NOS	<1	0
Basal-cell carcinoma	<1	<1
Ovarian cyst	<1	0
Chest pain	<1	<1
Cholelithiasis	<1	<1
Depression	<1	<1
Intervertebral disk herniation	<1	<1
Multiple sclerosis	<1	<1
Perforating appendicitis	<1	0
Colitis, NOS	<1	0
Dehydration	<1	0
Fall	<1	0
Abnormal liver-function values	<1	<1
Traffic accident	<1	0
Urinary tract infection, NOS	<1	<1
Uterine fibroids	<1	<1
Viral infection, NOS	<1	0
PML**	<1	0

* NOS denotes not otherwise specified, and PML progressive multifocal leukoencephalopathy.
† The adverse events listed are those that occurred at an incidence of at least 5 percent in the combination-therapy group and at an incidence of at least 1 percent in the combination-therapy group as compared with the group assigned to interferon beta-1a alone.
‡ $P \leq 0.01$ for the comparison with the group assigned to interferon beta-1a alone.
§ $P \leq 0.05$ for the comparison with the group assigned to interferon beta-1a alone.
¶ $P \leq 0.001$ for the comparison with the group assigned to interferon beta-1a alone.
‖ Serious adverse events were those that occurred in at least two patients (0.01 percent) in the combination-therapy group.
** Although there was only one case of PML during the Safety and Efficacy of Natalizumab in Combination with Interferon Beta-1a in Patients with Relapsing Remitting Multiple Sclerosis study, it is included in this table because of its importance and severity; a second, fatal case of PML was identified after the end of the two-year study.

increase in the incidence of chemical abnormalities, including the results of liver-function tests, was observed with combination therapy.

IMMUNOGENICITY

Seventy patients (12 percent of the combination-therapy group) had antibodies to natalizumab. Persistent antinatalizumab antibodies (detectable on at least two occasions 42 or more days apart) developed in 38 patients (6 percent), resulting in a loss of efficacy and an increase in infusion-related adverse events. The incidence of new neutralizing antibodies to interferon beta-1a was 1 percent among patients assigned to combination therapy and less than 1 percent among those assigned to interferon beta-1a alone.

DISCUSSION

Phase 3 trials have shown that over a two-year period, 62 to 75 percent of patients have clinical relapses while receiving interferon beta therapy.[5,6,8] For patients who have breakthrough disease while receiving disease-modifying therapies, clinical practice includes the addition of a second partially effective agent; however, there is no class I evidence to support this treatment strategy. The primary objective of SENTINEL was to address this common clinical scenario — specifically, to determine whether the addition of natalizumab to interferon beta-1a would reduce breakthrough disease activity in patients already receiving interferon beta-1a therapy. This approach has been used effectively in the development of combination therapy for rheumatoid arthritis.[29-32]

The addition of natalizumab to interferon beta-1a reduced the risk of disability progression by 24 percent over a two-year period as compared with interferon beta-1a alone (P=0.02). The sensitivity analysis of disability sustained for 24 weeks did not reach statistical significance (P=0.17); however, that analysis was exploratory, and the study was not adequately powered to assess the treatment effect on the basis of this definition. We also found that combination therapy reduced the annualized rate of relapse by 55 percent over a two-year period as compared with interferon beta-1a alone (P<0.001).

Accumulation of T_2-hyperintense MRI lesions has been linked to future progression of brain atrophy[33] and long-term disability[34,35] in relapsing multiple sclerosis. The number of new or enlarging T_2-hyperintense lesions in patients receiving interferon beta-1a alone was similar to the findings of another study of interferon beta-1a in relapsing multiple sclerosis that used the same imaging methods.[36] The addition of natalizumab to interferon beta-1a further reduced the number of new or enlarging T_2-hyperintense lesions by 83 percent, and approximately two thirds of the patients assigned to combination therapy remained free of new lesions for two years.

Natalizumab interferes with the activity of α_4 integrin, altering cell migration into the central nervous system and possibly blocking interactions between α_4 integrin and its ligands within the central nervous system itself.[1-4] Interferon beta has pleiotropic effects on cellular functions that are relevant to efficacy in multiple sclerosis and distinct from those of natalizumab.[11-14] In addition, studies have shown that, like natalizumab, interferon beta may prevent leukocyte migration across the blood–brain barrier by altering the expression of adhesion molecules.[15-17] The additional efficacy of the combination over that conferred by interferon beta alone suggests that the interaction between $\alpha_4\beta_1$ integrin and its targets is a key mediator of inflammation and subsequent demyelination in multiple sclerosis.

In February 2005, administration of natalizumab was suspended when two cases of PML were identified. In one of the cases, PML was diagnosed during SENTINEL, and in the other it was diagnosed after the patient had completed SENTINEL and had begun participating in an open-label safety study of natalizumab and interferon beta-1a. Later, an additional case of PML was identified post mortem in a patient with Crohn's disease who had previously received a diagnosis of astrocytoma. Details of these three cases have recently been published.[26,27,37] An extensive safety evaluation of patients in clinical trials who were receiving natalizumab at the time of the drug suspension did not identify additional cases of PML (see the article by Yousry et al. in this issue of the *Journal*[38]). The mechanisms by which natalizumab may increase the risk of PML are unknown, but they may involve altered trafficking of lymphoid cells harboring latent JC virus, decreased immune surveillance, or a combination of these processes.[39] The role of interferon beta in combination with natalizumab is also not clear, given that PML has never been associated with interferon beta alone.

SENTINEL was designed to determine whether natalizumab added to interferon beta-1a is better than interferon beta-1a alone. The results of all prespecified analyses of primary and secondary end points were positive and statistically significant. A natalizumab-monotherapy group was not included in the trial because this design would have required withdrawal of an approved therapy in order to switch to an experimental one at a time (in 2001) when the long-term safety and efficacy of natalizumab were unknown. This approach was believed to be unacceptable by the investigator advisory committee. Hence, additional studies would be required to determine whether combination therapy with natalizumab

and interferon beta-1a is more efficacious than natalizumab alone and to define further the role of natalizumab combination therapy in clinical practice. The results of another trial of natalizumab, administered without interferon beta-1a, also appear in this issue of the *Journal*.[40]

SENTINEL systematically evaluated combination therapy as compared with standard interferon beta therapy in relapsing multiple sclerosis. The study showed that in patients with multiple sclerosis who have breakthrough disease during interferon beta treatment, combination therapy has significant benefits when compared with interferon beta-1a alone. Additional studies will be required for further assessment of the long-term safety of combination therapy with natalizumab and for assessment of its efficacy relative to that of natalizumab alone.

Supported by Biogen Idec and Elan Pharmaceuticals.

Drs. Rudick and Stuart report having received consulting fees, lecture fees, and grant support from Biogen Idec. Dr. Calabresi reports having received consulting fees from Biogen Idec, Teva, Schering, and Novartis; lecture fees from Biogen Idec and Teva; and grant support from Biogen Idec and Genentech. Dr. Confavreux reports having received consulting and lecture fees from Biogen Idec, Sanofi-Aventis, Schering, Serono, and Teva. Dr. Galetta reports having received consulting fees, lecture fees, and grant support from Biogen Idec. Dr. Radue reports having received lecture fees from Biogen Idec. Dr. Lublin reports having received grant support from Biogen Idec, Teva, Acorda, and Merck and consulting or lecture fees from Biogen Idec, Berlex, Teva, Novartis, Schering-Plough, Serono, Pfizer, Amgen, and Antisense Therapeutics. Dr. Weinstock-Guttman reports having received lecture fees from Biogen Idec and Teva and grant support from Biogen Idec. Dr. Wynn reports having received consulting fees from Biogen Idec, Teva, Serono, and Avanir Pharmaceuticals and lecture fees from Biogen Idec, Teva, Pfizer, and Serono. Ms. Lynn, Dr. Panzara, and Dr. Sandrock report having equity interest in and being employees of Biogen Idec. No other potential conflict of interest relevant to this article was reported.

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Exhibit 2

BRIEF REPORT

Progressive Multifocal Leukoencephalopathy Complicating Treatment with Natalizumab and Interferon Beta-1a for Multiple Sclerosis

B.K. Kleinschmidt-DeMasters, M.D., and Kenneth L. Tyler, M.D.

SUMMARY

A 46-year-old woman with relapsing–remitting multiple sclerosis died from progressive multifocal leukoencephalopathy (PML) after having received 37 doses of natalizumab (300 mg every four weeks) as part of a clinical trial of natalizumab and interferon beta-1a. PML was diagnosed on the basis of the finding of JC viral DNA in cerebrospinal fluid on polymerase-chain-reaction assay and was confirmed at autopsy. Nearly every tissue section from bilateral cerebral hemispheres contained either macroscopic or microscopic PML lesions. There was extensive tissue destruction and cavitation in the left frontoparietal area, large numbers of bizarre astrocytes, and inclusion-bearing oligodendrocytes, which were positive for JC virus DNA on in situ hybridization.

PROGRESSIVE MULTIFOCAL LEUKOENCEPHALOPATHY (PML), A DEMYELINating disease of the central nervous system (CNS), is associated with high rates of morbidity and mortality and occurs almost exclusively in immunocompromised patients.[1] We describe a patient with multiple sclerosis who died of PML after receiving natalizumab (Tysabri, Biogen Idec) as part of a clinical trial conducted to test the safety and efficacy of natalizumab in combination with interferon beta-1a (Avonex, Biogen Idec) in the treatment of relapsing–remitting multiple sclerosis. To our knowledge, there have been no prior reports of the concomitant association of multiple sclerosis and PML.

Natalizumab is a humanized monoclonal antibody against α_4 integrins that was recently introduced for the treatment of multiple sclerosis. The drug was withdrawn from the market after reports of the development of PML in two patients with multiple sclerosis who were receiving natalizumab and interferon beta-1a in clinical trials. An additional case of PML, in a patient receiving natalizumab for the treatment of Crohn's disease, is described elsewhere in this issue of the *Journal*.[2]

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This article was published at www.nejm.org on June 9, 2005.

N Engl J Med 2005;353:369-74.

CASE REPORT

A 41-year-old, right-handed woman began to have numbness and burning pain in her right foot and leg and tingling numbness and clumsiness in her right hand in June 1999. She had a history of migraine and transient numbness of the left hand. A neurologic examination revealed increased tone on her right side and generalized hyperreflexia (3+) with normal plantar responses. In September 1999, magnetic resonance imaging (MRI) with contrast medium showed four small, nonenhancing foci of increased signal in the

corona radiata bilaterally on the fluid-attenuated inversion recovery (FLAIR) sequences. Electromyography and nerve-conduction studies showed no abnormalities. Six weeks later, her leg symptoms had improved, but the patient reported new visual blurring in her right eye. The visual acuity of the right eye was 20/100, and that of the left was 20/15. Examination of cerebrospinal fluid in November 1999 showed 1 white cell per cubic millimeter, 55 mg of protein per deciliter, and normal values for glucose (64 mg per deciliter [3.6 mmol per liter]), IgG (3.2 mg per deciliter), the IgG index (0.57), and the IgG-synthesis rate (0.3 mg per 24 hours). No oligoclonal bands were detected in a specimen of cerebrospinal fluid that was concentrated by a factor of 80. Levels of vitamin B_{12} and folate were normal, tests for antinuclear antibodies and anticentromere antibodies were negative, and thyroid-function tests were normal. A complete blood count was also normal, except for mild leukocytosis (11,200 cells per cubic millimeter).

In January 2000, MRI showed two new nonenhancing parietal lesions with increased FLAIR signal and decreased T_1-weighted signal. In February 2000, the patient reported that her vision was normal and that her right-sided numbness had nearly resolved. She began receiving 30 μg of interferon beta-1a intramuscularly each week, tizanidine, calcium, magnesium, and vitamins B, C, and E for presumed multiple sclerosis (Table 1). In May 2000, she began taking tamsulosin for difficulty with bladder emptying and citalopram for depression.

In March 2001, the patient noted worsening vision, band-like paresthesias around her back and abdomen, and increasing weakness and spasticity of her legs. The strength of both legs was mildly decreased (4+/5), and her gait was slightly spastic, although her deep tendon reflexes were normal. She received 500 mg of methylprednisolone twice daily intravenously for five days (March 16 through 20, 2001) for a suspected exacerbation of multiple sclerosis. In September 2001, she reported some decline in fine motor skills in her hands and worsening spasticity in her legs as well as some decline in cognition, including short-term memory, and began taking donepezil. She had a score of 2.5 on the Kurtzke Expanded Disability Status Scale (EDSS) in March 2002 (range of scores, 0 to 10, with higher scores indicating a greater degree of disability).

In April 2002, the patient was enrolled in a randomized, placebo-controlled, parallel-group, multicenter study designed to determine the safety and efficacy of natalizumab combined with interferon beta-1a in patients with relapsing–remitting multiple sclerosis (the Biogen Idec and Elan 1802 SENTINEL trial). At the time of her enrollment, T_2-weighted MRI showed approximately nine lesions and her EDSS score was 0. She continued to take 30 μg of interferon beta-1a intramuscularly weekly throughout the study. Additional medications at study entry included citalopram, rofecoxib, and tramadol in combination with acetaminophen.

During the study, the patient received a total of 30 doses of natalizumab (300 mg, or approximately 6 mg per kilogram of body weight, each) by intravenous infusion at four-week intervals between April 12, 2002, and July 9, 2004. She also received tizanidine, donepezil, and briefly, galantamine. In July 2004, she was enrolled in an open-label extension study (Biogen Idec/Elan 1808) and received seven additional 300-mg doses of natalizumab at four-week intervals, with the last dose given on January 18, 2005. No antibodies developed against either interferon beta-1a or natalizumab. Pharmacokinetic studies showed that the clearance of natalizumab in the patient (0.0136 liter per hour) was similar to the median value in the study population (0.0138 liter per hour). A follow-up T_2-weighted MRI study in April 2003 showed five new or enlarging lesions. A similar study in April 2004 showed one new or enlarging lesion. No enhancing lesions were noted. Unfortunately, these MRI scans were not available for review, and the reports specified only the number of lesions, not the location. No clinical or suspected relapses of multiple sclerosis were identified, and the patient's EDSS score remained between 0 and 2 through July 2004.

In November 2004, the patient reported new problems with hand–eye coordination, including difficulty writing and typing, as well as problems with her speech. A mental-status examination performed at that time showed a decreased fund of in-

Table 1. Doses and Timing of Treatments for Multiple Sclerosis.

Treatment*	Treatment Interval
Interferon beta-1a, 30 μg IM	February 2000–January 2005
Methylprednisolone, 500 mg IV twice daily	March 16–20, 2001 December 15–19, 2004 January 5–9, 2005
Natalizumab, 300 mg IV every 4 weeks	April 12, 2002–January 18, 2005

* IM denotes intramuscularly, and IV intravenously.

formation, minor errors on a drawing of a three-dimensional cube and on tests of mathematical skills, and reduced immediate recall on a word-learning test. Her cranial nerves were normal. Her strength was intact, but she had mildly increased tone in her legs and hyperactive (3+) reflexes bilaterally. In December 2004, right-sided numbness developed and word-finding difficulty increased. The patient had difficulty carrying on a conversation and became increasingly forgetful. A neurologic examination revealed difficulty with expressive speech, with preserved comprehension, some right–left confusion, irregular saccadic eye movements, and increased tone on her right side. She received methylprednisolone (500 mg intravenously twice daily) from December 15 through 19, 2004. An MRI study performed on December 15 showed a large area of increased T_2-weighted and decreased T_1-weighted signal in the left frontal lobe posteriorly involving the subcortical white matter and extending into the centrum semiovale and corona radiata, without enhancement or mass effect (Fig. 1A). A second area of abnormal signal was noted in the right posterior parietal lobe. On December 29, 2004, a right-sided hemiparesis with an extensor plantar response was noted.

On January 5, 2005, the patient's condition was judged to be worse, with increasing right-sided hemiparesis and worsening nonfluent aphasia. Her right-arm strength was 0/5, and her right-leg strength was 2/5 proximally and 0/5 distally. On the assumption that her clinical deterioration represented an exacerbation of multiple sclerosis, she received another five-day course of methylprednisolone, beginning on January 5, 2005 (500 mg intravenously twice daily). Her last dose of natalizumab was given on January 18, 2005. On January 24, 2005, her white-cell count was 14,400 per cubic millimeter (77 percent neutrophils, 18 percent lymphocytes, 4 percent monocytes, and 1 percent eosinophils), with an absolute lymphocyte count of 2500 per cubic millimeter.

The patient's neurologic status continued to decline, and she was hospitalized on February 12, 2005. On admission, she was unresponsive, with a right-sided gaze preference. She had a marked spastic right-sided hemiplegia and some left-sided weakness. An MRI scan obtained on February 12 (Fig. 1B and 1C) showed a dramatic increase in the extent of the high T_2-weighted and low T_1-weighted signal abnormalities in the left hemisphere, with extension of the lesion to the frontal, parietal, and temporal lobes and across the corpus callosum to the right frontal lobe. New midbrain and pontine lesions were also present. There was no enhancement or mass effect. At admission, the patient had a white-cell count of 14,000 per cubic millimeter (77 percent neutrophils, 16 percent lymphocytes, 6 percent monocytes, and 1 percent eosinophils and basophils). Her absolute lymphocyte count was normal (2300 cells per cubic millimeter).

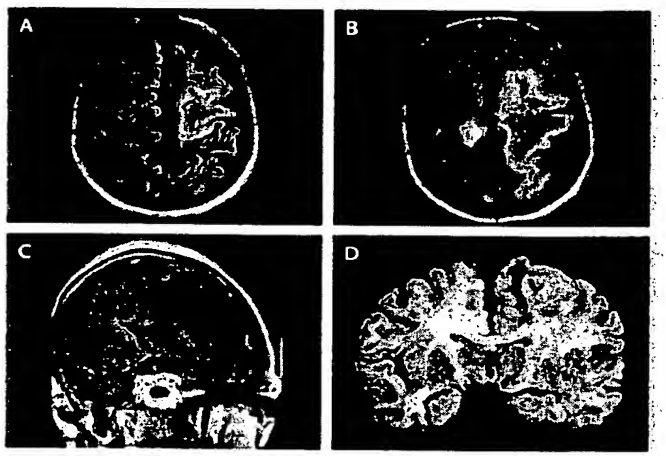

Figure 1. MRI Findings (Panels A, B, and C) and Autopsy Findings (Panel D).
In Panel A, a fast spin–echo inversion recovery sequence (repetition time, 9000 msec; echo time, 91 msec; inversion time, 2500 msec) from MRI performed on December 15, 2004, shows a large PML lesion in the left frontal lobe. In Panel B, a fast spin–echo inversion recovery sequence (repetition time, 10,002 msec; echo time, 145 msec; inversion time, 2200 msec) from MRI performed on February 12, 2005, shows an increase in the size of the previously noted lesion as well as new PML lesions in the parietal and occipital lobes in the opposite hemisphere. In Panel C, a fast spin–echo sequence (repetition time, 516 msec; echo time, 10.9 msec) from MRI performed on February 12, 2005, shows a large, low-signal PML lesion underlying the cortex in the left frontoparietal white matter. For comparison with Panel C, in Panel D, a formalin-fixed coronal section of the brain shows massive coalescent areas of cavitation of the left frontoparietal white matter, leaving only a ribbon-like strip of intact overlying cortex.

An examination of cerebrospinal fluid on February 14, 2005, revealed the following values: 53 mg of protein per deciliter, 90 mg of glucose per deciliter (5.0 mmol per liter), 4.3 mg of IgG per deciliter, an IgG index of 0.49, a ratio of IgG to albumin of 0.08, and an IgG-synthesis rate of 1.78 mg per 24 hours. No oligoclonal bands were noted. The results of Gram's staining of a cerebrospinal fluid sample were unremarkable. A polymerase-chain-reaction (PCR) assay of cerebrospinal fluid for herpes sim-

plex virus was negative, as were tests for West Nile virus IgG and IgM, eastern equine encephalomyelitis virus IgG and IgM, *Borrelia burgdorferi* IgG and IgM, and cryptococcal antigen and stains and cultures for bacteria, fungi, and acid-fast bacilli. A test for serum antibody against human immunodeficiency virus (HIV) type 1 and 2 was nonreactive. CD4+ and CD8+ T-cell counts were not assessed, but at no time was either absolute or relative lymphopenia noted.

The treating neurologist suspected PML, and a cerebrospinal fluid sample sent to the Mayo Medical Laboratories (Rochester, Minn.) for JC virus PCR testing was positive. The patient died on February 24, 2005; she was 46 years old.

METHODS AND RESULTS

Postmortem examination showed bilateral aspiration pneumonia and cachexia. There was prominent sinus histiocytosis of the lymph nodes and possible depletion of CD8+ T cells in comparison with the levels of CD4+ T cells, probably owing to severe terminal debilitation. Examination of the bone marrow showed a clinically significant leftward shift in granulocytic maturation. All other systemic organs were histologically normal; no non-CNS opportunistic infections were found. Postmortem blood samples were not tested for JC virus DNA or antibody.

The formalin-fixed, 1140-g brain was fluctuant on palpation over a large portion of the anterior left hemisphere; no discoloration or meningeal opacification was present. On coronal sectioning, this softened area corresponded to massive, coalescent areas of severe cavitation involving most of the left frontoparietal white matter, leaving only a ribbon-like strip of intact overlying cortex (Fig. 1D). Smaller, noncavitated, ovoid areas of discoloration, a typical feature of PML, studded the remaining left-hemispheric white matter, particularly at the junctions between cortical gray matter and white matter, and involved the right superior frontal gyrus (Fig. 2A). A 7-mm lesion was identified in the left cerebral peduncle (Fig. 2B and 2C). No multiple-sclerosis plaques were discernible in the corona radiata.

The brain stem, spinal cord, and optic chiasm were submitted in toto for histologic examination. Sections (total, 73 blocks) from the brain and spinal cord were stained with hematoxylin and eosin, with one fourth of the sections also stained with Luxol fast blue and periodic acid–Schiff for myelin. All sections were devoid of acute anoxic injury and vasculitis. Areas of PML showed near-total loss of myelin, an influx of macrophages, and numerous reactive astrocytes, but no perivascular or parenchymal lymphocytic inflammation (Fig. 2D). Astrocytes with bizarre, enlarged hyperchromatic nuclei, a typical finding in PML, were common, even in smaller lesions (Fig. 2D). There were large numbers of oligodendrocytes with the classic violaceous intranuclear inclusions of PML (Fig. 2E). Cells with inclusions had a strong positive signal for JC virus DNA on in situ hybridization (probe 40847, Enzo Life Sciences) (Fig. 2F).

In addition to the PML lesions seen on gross examination, myriad minute lesions were easily identified microscopically in virtually every section examined from the left cerebral hemisphere, as well as in most of the sections from the right side and all of the brain-stem sections. PML was found only focally in the cerebellum; no granule-cell depletion was seen. The optic nerve, chiasm, and spinal cord contained neither PML lesions nor multiple-sclerosis demyelinating lesions. Examination of the spinal cord showed unilateral wallerian degeneration that was due to the cavitated lesions involving the left motor strip and internal capsule. Remote cortical microinfarctions were found in the right superior frontal and parietal gyri and splenium of the corpus callosum.

DISCUSSION

PML is a demyelinating disease of the CNS caused by lytic infection of oligodendrocytes by JC polyomavirus. Primary JC virus infection occurs in childhood and is asymptomatic. JC virus antibodies are detectable in approximately 50 to 70 percent of the adult population.[3,4] After the primary infection, JC virus remains latent in kidneys and lymphoid organs. Up to 64 percent of healthy adults have shedding of JC virus in urine in the absence of any clinical symptoms, suggesting that asymptomatic active JC virus infection is common in immunocompetent persons.[5] In contrast, PML occurs almost exclusively in immunocompromised persons, particularly those with depressed cell-mediated immunity resulting from HIV infection, hematologic cancers, or immunosuppressive medications.[1] In recipients of bone marrow transplants, PML has also been associated with treatment with rituximab, an antibody against CD20 expressed on B cells,[6] and cases of PML-like CNS demyelinating illness have been reported in patients with rheumatic diseases treated

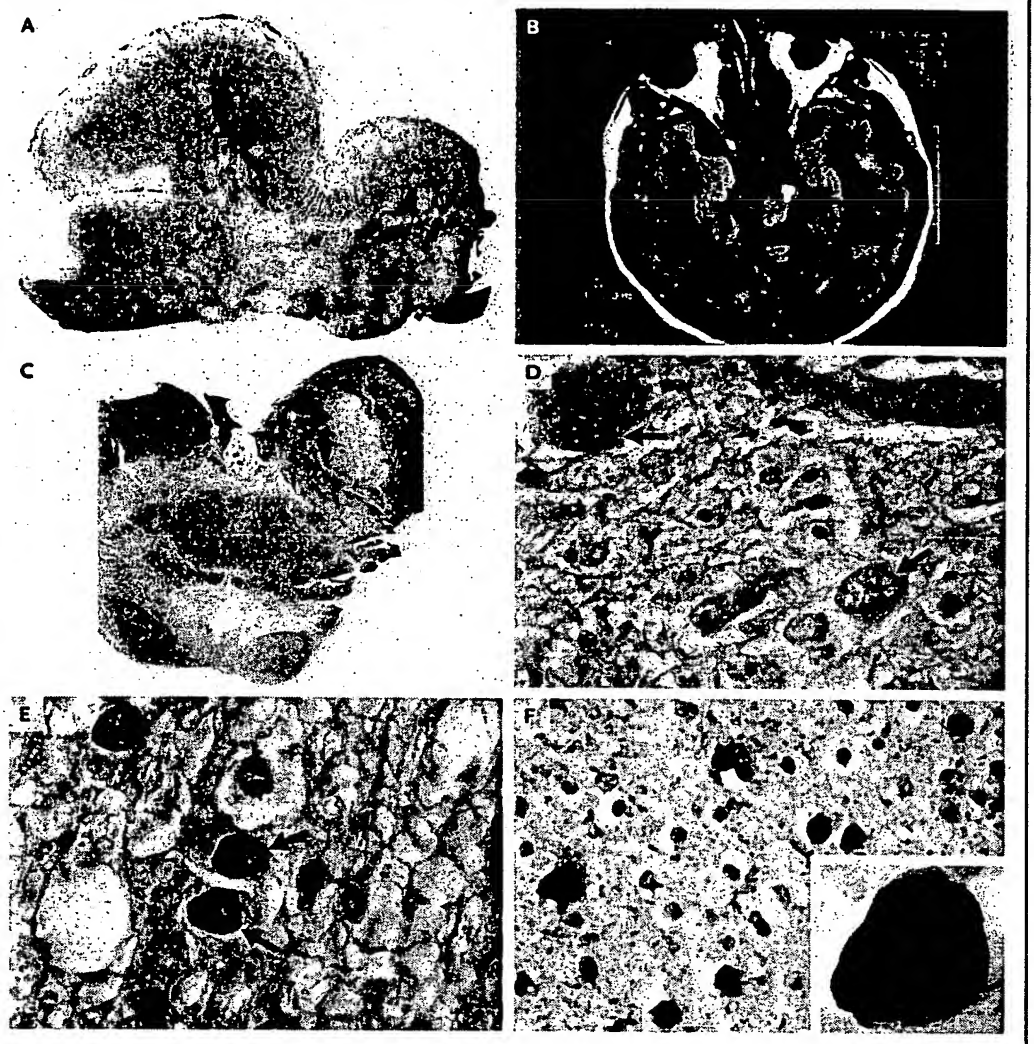

Figure 2. Histologic and MRI Findings.

Panel A shows smaller, noncavitated, ovoid areas of discoloration typical of PML studding the left-hemispheric white matter, particularly at the junctions of cortical gray matter and white matter, as well as the right superior frontal gyrus (whole-mount section stained for myelin with Luxol fast blue–periodic acid–Schiff). In Panel B, a fast spin–echo inversion recovery sequence (repetition time, 10,002 msec; echo time, 145 msec; and inversion time, 2200 msec) from MRI performed on February 12, 2005, shows PML lesions in the left cerebral peduncle of the midbrain, left temporal lobe, and both occipital lobes. For comparison with Panel B, Panel C shows a discrete PML lesion, 7 mm in diameter, in the left cerebral peduncle (whole-mount section stained for myelin with Luxol fast blue and periodic acid–Schiff). In Panel D, PML lesions are characterized by a near-total loss of myelin, an influx of macrophages, and numerous bizarre astrocytes (arrows), but no perivascular or parenchymal lymphocytic inflammation (hematoxylin and eosin). Panel E shows large numbers of oligodendrocytes with the violaceous intranuclear inclusions characteristic of PML; several infected glial cells are also present (arrows) (hematoxylin and eosin). In Panel F and the inset, cells with inclusions have a strong positive nuclear signal for JC virus (dark reddish brown) of PML on in situ hybridization (diaminobenzidine used as the chromagen with a light hematoxylin counterstain).

with antagonists of tumor necrosis factor α.[7] Although multiple sclerosis is an immune-mediated disorder, to our knowledge, patients with multiple sclerosis have not previously been identified as at increased risk for PML.

Natalizumab is a humanized monoclonal antibody against α_4 integrins that was approved by the Food and Drug Administration for the treatment of several immune-mediated disorders, including multiple sclerosis and inflammatory bowel disease.[8-10] Antibodies against α_4 integrins inhibit the binding of cells expressing $\alpha_4\beta_1$ integrin and $\alpha_4\beta_7$ integrin (e.g., lymphocytes) to vascular-cell adhesion molecule 1 and mucosal addressin-cell adhesion molecule 1 on endothelial cells, a critical step in the diapedesis of lymphocytes across blood vessels into the CNS and mucosal organs.[10,11] Treatment with antibodies against α_4 integrins prevents inflammatory cells from crossing the blood–brain barrier and inhibits the accumulation of immune cells in the CNS in animals with experimental allergic encephalomyelitis.[11-13]

Our patient received interferon beta-1a for nearly five years and received combined therapy with natalizumab and interferon beta-1a for just over two years as part of the SENTINEL trial. We therefore cannot rule out a potential contributory role of interferon beta-1a in the genesis of this patient's PML. However, to date, there have been no reported cases of PML in patients receiving interferon beta-1a monotherapy.

The diagnosis of PML was established on the basis of a positive PCR assay for JC viral DNA in cerebrospinal fluid in a patient with clinical and neuroimaging findings that were typical of PML, and the diagnosis was confirmed at autopsy. The severity and extent of disease were dramatic. Nearly every tissue section from bilateral cerebral hemispheres that we examined had either macroscopic or microscopic PML lesions, ranging from minute to massive in size. There was extensive tissue destruction and cavitation in the left frontoparietal area, and the lesions contained large numbers of oligodendrocytes with inclusions. No inflammatory response was present. Although no formal quantitation was performed, the extent of the PML involvement was similar to or exceeded that seen in HIV-infected patients before the advent of highly active antiretroviral therapy.

Dr. Tyler is supported by the Reuler–Lewin Family Professorship of Neurology.

We are indebted to the neurologist who cared for the patient; to Dr. Kate Dawson from Biogen Idec, for furnishing some additional clinical and laboratory information on the patient; to Ms. Lisa Litzenberger for her photographic expertise; to the histologic technicians, supervised by Mr. David Davis, for slide preparation; and to Ms. Cindy McNair for assistance in the preparation of the manuscript.

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Exhibit 3

BRIEF REPORT

Progressive Multifocal Leukoencephalopathy in a Patient Treated with Natalizumab

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SUMMARY

We describe the clinical course of a patient with multiple sclerosis in whom progressive multifocal leukoencephalopathy (PML), an opportunistic viral infection of the central nervous system, developed during treatment with interferon beta-1a and a selective adhesion-molecule blocker, natalizumab. The first PML lesion apparent on magnetic resonance imaging was indistinguishable from a multiple sclerosis lesion. Despite treatment with corticosteroids, cidofovir, and intravenous immune globulin, PML progressed rapidly, rendering the patient quadriparetic, globally aphasic, and minimally responsive. Three months after natalizumab therapy was discontinued, changes consistent with an immune-reconstitution inflammatory syndrome developed. The patient was treated with systemic cytarabine, and two months later, his condition had improved.

PROGRESSIVE MULTIFOCAL LEUKOENCEPHALOPATHY (PML) IS A RARE, OLIgodendroglial infection caused by the polyomavirus JC virus. It usually occurs in people infected with the human immunodeficiency virus (HIV), but it has also been reported in immunocompromised patients receiving prolonged treatment with methotrexate, cyclophosphamide, and azathioprine. PML has not been reported in persons with multiple sclerosis, despite the frequent use of these medications to treat the disease.

We describe the clinical course of a patient with multiple sclerosis in whom PML developed during treatment with interferon beta-1a (Avonex, Biogen Idec) and natalizumab (Tysabri, Biogen Idec and Elan), a monoclonal antibody against α_4 integrins. Despite the discontinuation of these medications, his PML progressed rapidly. An immune-reconstitution inflammatory syndrome developed three months after the cessation of natalizumab therapy, and the patient became bedridden and minimally responsive. Treatment with intravenous cytarabine was begun, and shortly thereafter, his condition improved. The reasons for his clinical deterioration and recovery are not clear.

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This article was published at www.nejm.org on June 9, 2005.

N Engl J Med 2005;353:375-81.

CASE REPORT

In 1983, a 23-year-old right-handed man had a month-long episode of right hemianesthesia, his first symptom of what proved to be relapsing–remitting multiple sclerosis. He had a second attack in 1989 and had two or three attacks per year between 1989 and 1998. His medical history was also notable for the Ramsay Hunt syndrome with auricular zoster in 1998, a malignant melanoma excised from his back with negative margins in 1996, and a cleft lip and palate. A sister also had relapsing–remitting multiple sclerosis.

He started receiving weekly intramuscular injections of interferon beta-1a in 1998 (Fig. 1). The frequency of relapses decreased to one per year until 2001. From 2001 through 2002 he had three exacerbations, prompting his enrollment in a double-blind, randomized, placebo-controlled trial of 300 mg of natalizumab every four weeks plus interferon beta-1a as compared with a placebo infusion plus interferon beta-1a. At entry into the study in October 2002, he had an old left afferent pupillary deficit, mild right lateral rectus palsy, right-sided lower-motor-neuron facial paresis, mild ataxia, a score on the Kurtzke Expanded Disability Status Scale of 2 (scores can range from 0 to 10, with higher scores indicating more severe disease), and evidence of focal, nonenhancing white-matter lesions on T_2-weighted magnetic resonance imaging (MRI) characteristic of multiple sclerosis. During the next two years he had no further relapses. T_2-weighted MRI of the brain, performed as part of the study protocol in October 2003, showed multiple small, nonenhancing periventricular and subcortical hyperintensities consistent with the presence of multiple sclerosis. But in October 2004, in addition to a small, new, enhancing periventricular lesion typical of multiple sclerosis (not shown), a new nonenhancing lesion of the right frontal lobe appeared on another MRI scan obtained as part of the protocol (Fig. 2A).

In November 2004, the patient's physician observed uncharacteristic, inappropriate behavior during a routine study visit. In mid-December, the patient told his family and friends that he was having difficulty with attention and concentration. Progressive left hemiparesis, dysarthria, and cognitive impairment subsequently developed. MRI of the brain showed new, extensive abnormalities, including a large hyperintense lesion of the right frontal lobe, bilateral subinsular white-matter lesions that spared the cortex, and scattered lesions in the white matter, deep gray matter, and brain stem, with a few punctate foci of enhancement consistent with the presence of noninflammatory PML[1] (Fig. 2B). After receiving 28 infusions, the last in mid-December 2004, the patient stopped taking the study drug, which was revealed to be natalizumab.

The patient was not classically immunocompromised at clinical presentation: he had no known risk factors for HIV infection, serologic analysis for HIV was twice negative, and the total leukocyte count (8.6×10^3 per cubic millimeter) and values for lymphocyte subgroups were normal (CD4:CD8 ratio, 1.1; CD4 T-cell count, 637 per cubic millimeter; and CD8 T-cell count, 564 per cubic millimeter). Analysis of cerebrospinal fluid in early February showed no white cells and 88 red cells per cubic millimeter, normal cytologic findings, and normal concentrations of both total protein (41 mg per deciliter) and glucose (62 mg per deciliter [3.4 mmol per liter]). The IgG index (a measure of the IgG production in the cerebrospinal fluid) was elevated (0.7), and two oligoclonal bands were seen. JC virus DNA was detected by the polymerase chain reaction (PCR)[2] in the serum (2500 copies per milliliter), peripheral-blood mononuclear cells (225 copies per milliliter), and cerebrospinal fluid (6050 copies per milliliter). Biopsy of the right frontal lobe showed abundant areas of astrogliosis and microgliosis in the deep layers of cortical gray matter, with underlying white matter showing demyelination, dense infiltration of macrophages, and sparse lymphocytes. Scattered enlarged oligodendrocytes contained intranuclear inclusions positive for papovavirus (Fig. 3). In situ hybridization showed JC virus but no evidence of herpes simplex virus or cytomegalovirus. A workup for cancer, including computed tomography (CT) of the chest, abdomen, and pelvis and whole-body positron-emission tomography, showed no masses and no areas of increased metabolism. Positron-emission tomography did show decreased cortical uptake of fludeoxyglucose F 18 within the right frontal lobe, a finding consistent with necrosis.

During the next three weeks, left hemiplegia, left-sided neglect, left hemianesthesia, apraxia of the right arm, and nonfluent aphasia developed and dysarthria worsened despite intravenous treatment with high-dose methylprednisolone. Intravenous treatment with cidofovir (5 mg per kilogram of body weight every two weeks) was initiated.

Eight days later, global aphasia, incontinence, stooped posture, and truncal instability developed. Repeated analysis of cerebrospinal fluid showed a mild pleocytosis and hemorrhage: an elevated protein concentration (58 mg per deciliter), 2 white cells and 324 red cells in the second tube obtained, and 6 white cells (30 percent neutrophils, 55 percent lymphocytes, 4 percent reactive lymphocytes, and 11 percent monocytoid cells) and 913 red cells in the subsequent tube. JC virus DNA was undetectable in peripheral-blood mononuclear cells and plasma but remained present in the cerebrospinal

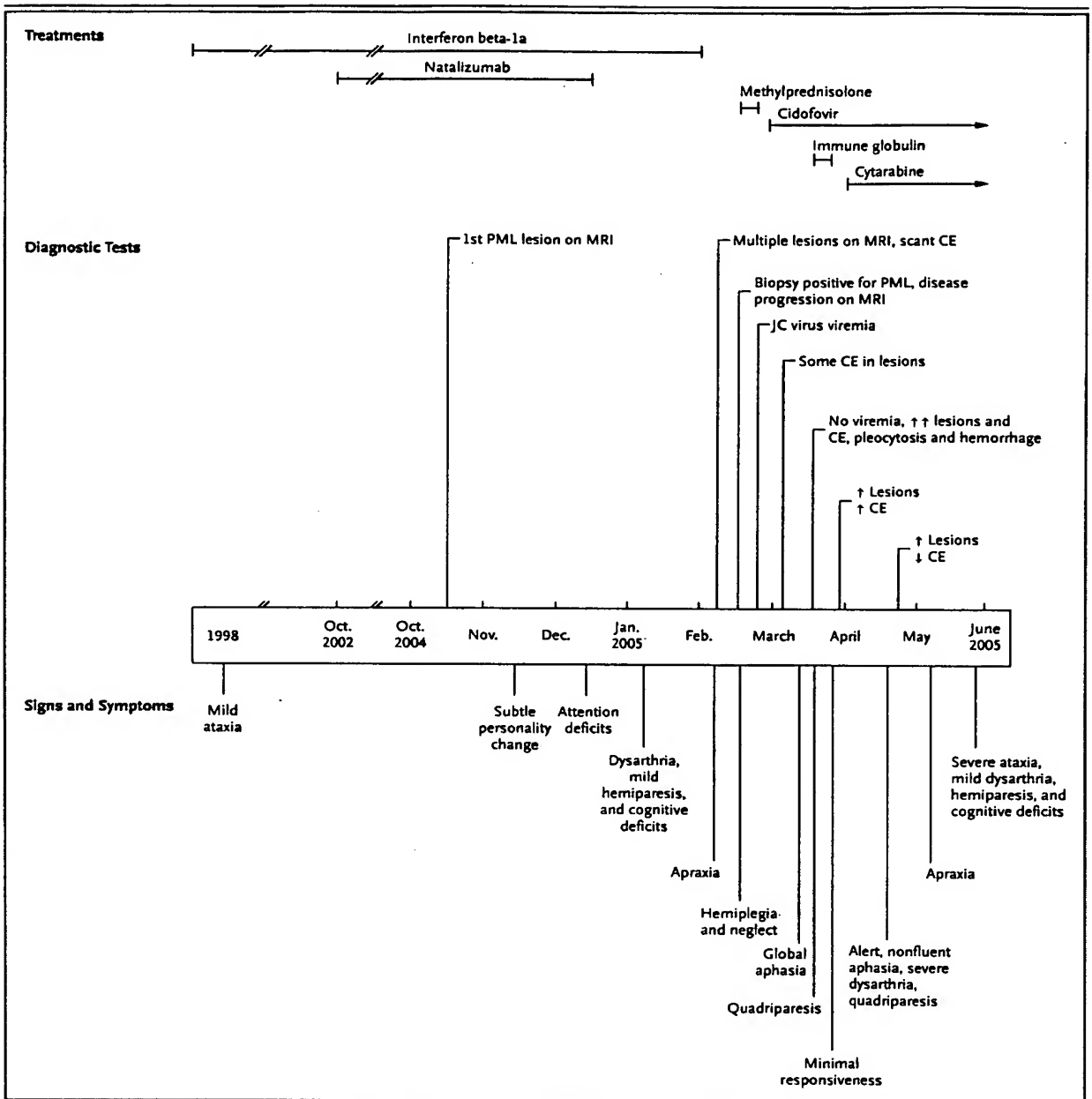

Figure 1. Summary of the Patient's Clinical Course, Treatments, and Results of Laboratory Tests.

CE denotes contrast enhancement. One upward-pointing arrow indicates a moderate increase, two upward-pointing arrows a substantial increase, and one downward-pointing arrow a moderate decrease.

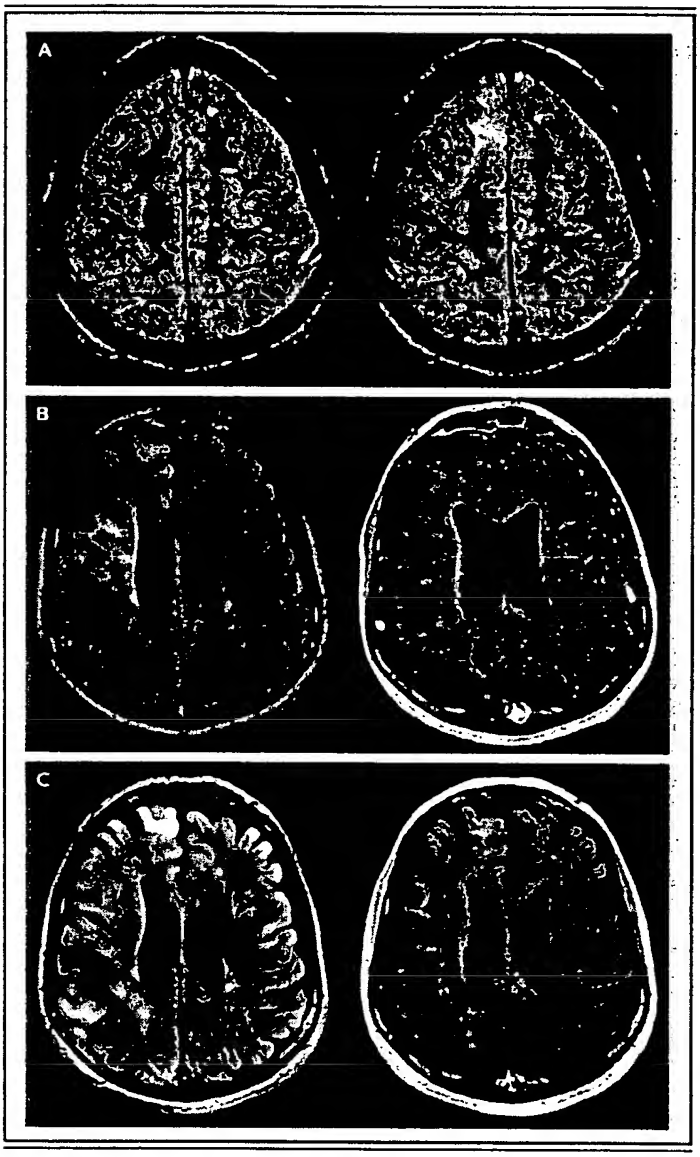

Figure 2. Progression of Abnormalities on MRI.

Panel A shows images obtained before the development of PML-related symptoms. An axial T_2-weighted MRI obtained in October 2003 (left-hand side) shows multiple small focal lesions in the white matter consistent with the presence of multiple sclerosis. In October 2004 (right-hand side), a large, new, ill-defined lesion is seen in the right frontal lobe, which will later prove to be PML. In early February 2005 (Panel B), axial fluid-attenuated inversion recovery MRI (left-hand side) shows more extensive disease in the right frontal white matter, with cortical sparing and several scattered lesions. After the addition of intravenous contrast medium (right-hand side), a few small foci of enhancement are apparent in the right hemisphere. In late March 2005 (Panel C), axial fluid-attenuated inversion recovery MRI (left-hand side) shows dramatic progression, especially in the right hemisphere, with lesions now extending into the anterior corpus callosum. After the addition of intravenous contrast medium (right-hand side), there is a substantial increase in the foci of enhancement.

fluid (2245 copies per milliliter). PCR of cerebrospinal fluid for herpes simplex virus, human herpesvirus 6, varicella–zoster virus, Epstein–Barr virus, and enteroviruses was negative, as were the results of Gram's staining, bacterial culture, cryptococcal staining, staining for acid-fast bacilli, and serologic analysis for Lyme disease. CT of the head showed no evidence of hemorrhage. MRI of the brain five weeks later (Fig. 2C) showed marked progression of the white- and gray-matter lesions and extensive foci of enhancement, particularly in the right hemisphere, findings consistent with inflammation.

The patient's hospital course was further complicated by methicillin-resistant *Staphylococcus aureus* bacteremia, urosepsis, upper gastrointestinal bleeding, elevated concentrations of serum aminotransferases, transient hyponatremia, and transient lymphopenia. The nadir absolute lymphocyte count was 647 cells per cubic millimeter, with 188 CD4+ T cells per cubic millimeter, 214 CD8+ T cells per cubic millimeter, and a CD4:CD8 ratio of 0.9.

His condition continued to deteriorate, despite the administration of three infusions of cidofovir over a period of eight weeks and a five-day course of intravenous immune globulin (2 g per kilogram per day). Left hemiplegia, anesthesia, and neglect were now accompanied by right hemiparesis and apraxia, nonfluent aphasia, severe cognitive impairment, and a fluctuating level of alertness, rendering the patient bedridden, mute, and almost completely noncommunicative. Electroencephalography at this time showed diffuse slowing and bilateral periodic epileptiform discharges that did not respond to treatment with intravenous benzodiazepam.

His treating physicians began intravenous treatment with cytarabine (2 mg per kilogram per day for five days) in early April. This caused pancytopenia, requiring the administration of erythropoietin and granulocyte colony-stimulating factor, and fever; the latter resolved within 12 hours after empirical antibiotic treatment.

Unexpectedly, the patient began talking two weeks after the initiation of cytarabine therapy. At the time of the most recent follow-up assessment, he continued to show neurologic improvement. After one month of cytarabine therapy, his right-sided weakness and left-sided sensory loss resolved, and his left hemiplegia, neglect, aphasia, and dysarthria began to improve. He still had severe deficits, including dysarthria, spastic left hemiparesis, cognitive impairment, and parkinsonism. He required the assistance of two persons to move from a bed to a chair. MRI of the brain obtained three weeks after treatment with cytarabine was begun showed further progression of disease in the left cerebellar white matter, right external and internal capsule, and frontal lobes bilaterally. The only detectable improvement was a slight decrease in the amount of contrast enhancement.

A second course of cytarabine was given four weeks after the first, without any complications. By the end of May 2005, the patient was starting to walk and was having meaningful conversations regarding the reasons for his clinical deterioration. He still had disabling ataxia, cognitive impairment, mild neglect, and mild left hemiparesis.

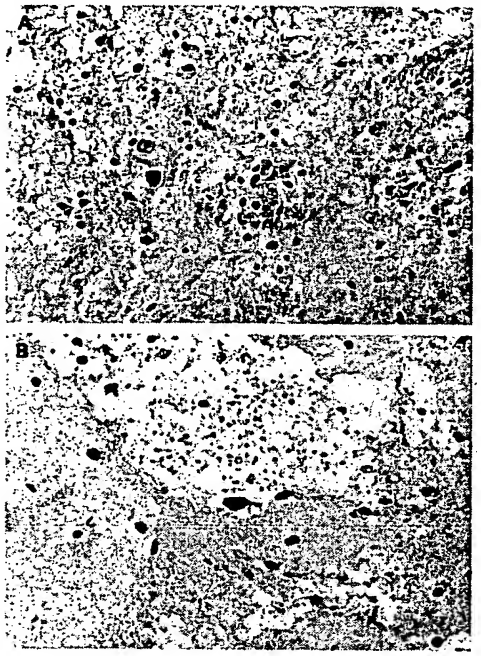

Figure 3. Brain-Biopsy Specimen.
Panel A shows a focus of demyelination (hematoxylin and eosin), and Panel B immunohistochemical staining for papovavirus.

DISCUSSION

Our patient is one of three patients in whom rapidly progressive PML has been shown to develop during clinical trials of natalizumab, a selective adhesion-molecule blocker, to treat relapsing–remitting multiple sclerosis or Crohn's disease.[3-5] Elsewhere in this issue of the *Journal*, Kleinschmidt-DeMasters and Tyler describe a second patient with multiple sclerosis who received combination treatment with natalizumab and interferon beta-1a[3] and Van Assche et al. describe a patient with Crohn's disease who received natalizumab alone.[4]

Our patient's condition worsened after the cessation of natalizumab therapy despite treatment with cidofovir, corticosteroids, and intravenous immune globulin, but his condition improved after the institution of systemic cytarabine therapy. His brain biopsy showed typical noninflammatory PML; however, three months after the cessation of natalizumab, what we believe to be an immune-reconstitution inflammatory syndrome developed that was characterized by widespread inflammation of the central nervous system, as shown by extensive enhancement on MRI and microscopic hemorrhages. Other remarkable features of the case include JC virus viremia and MRI evidence of PML one month before symptoms developed.

JC virus is a ubiquitous infection acquired in childhood that remains dormant in bone marrow, kidney epithelia, and spleen. Antibodies against JC virus are detectable in at least 80 percent of adults.[6] However, humoral immunity is insufficient to prevent the spread of the virus to the central nervous system. Intermittent reactivation, with shedding of live virus in the urine, has been well documented in cross-sectional studies of healthy adults and pregnant women, but this phenomenon is poorly understood. Spread of the virus to the central nervous system and the subsequent development of PML occur in immunocompromised persons — most commonly those infected with HIV, but also in some patients with lymphoma, sarcoidosis, and medication-induced immunosuppression. JC virus can enter the central nervous system directly during periods of viremia, such as those occurring during prolonged immunosuppression. Eighty

to 90 percent of patients with PML but not HIV infection die within one year.[7]

Natalizumab is highly effective at preventing recurrent inflammation in patients with multiple sclerosis.[8] Natalizumab binds to and blocks the function of α_4 integrins, adhesion molecules that promote the migration of lymphocytes into various organs, including the brain[9] and kidneys.[10] In patients with multiple sclerosis, natalizumab's most striking effect is the reduction of both contrast-enhancing lesions on MRI and clinical relapses.[8]

How natalizumab therapy alone or in combination with other immune-altering therapies could lead to JC virus viremia and PML is unknown. We speculate that the reactivation of the virus cannot be suppressed until the effects of natalizumab wear off. In our patient, JC virus viremia ended three months after treatment with natalizumab was stopped, and the biologic effects of natalizumab have been shown to wear off after about three months.[11]

Three months after natalizumab therapy was stopped, an inflammatory reaction developed in our patient's brain. In HIV-infected patients, as in our patient, inflammatory reactions against PML are a manifestation of the immune-reconstitution inflammatory syndrome and are associated with clinical deterioration and increases in the size of high signal lesions on T_2-weighted MRI but more favorable outcomes than in noninflammatory PML.[12,13] However, patients can die during the course of the immune-reconstitution inflammatory syndrome,[13] as our patient almost did, and how best to manage the JC virus infection and this inflammatory phase of PML is unknown.

Cidofovir, an antiviral agent, has been used with anecdotal success in the treatment of HIV-associated PML.[14,15] However, in vitro, cidofovir fails to kill glial cells infected with JC virus,[16] and there are no controlled studies to support its use. After three courses of cidofovir, our patient's condition continued to deteriorate.

Cytarabine kills JC virus in vitro.[16] This observation led to a randomized, controlled trial of the drug in HIV-infected patients with PML, which failed to show efficacy.[17] However, the penetration of cytarabine into the central nervous system is poor, and only one patient in this trial had contrast enhancement on MRI.[18] We chose to administer cytarabine to our patient, given the failure of cidofovir and the lack of other options, and subsequently, his condition improved. The reasons for this improvement are not clear. It is possible that the extensive breakdown of his blood–brain barrier improved penetration of cytarabine into the central nervous system, aiding in the clearance of the virus, or that its strong myelosuppressive properties curbed the inflammatory response. Alternatively, the improvement may have been due solely to clearance of the virus by the patient's reconstituted immune system.

In our patient, the first PML lesion — a frontal-lobe lesion that was indistinguishable from a multiple sclerosis lesion — was visible on neuroimaging studies two months before obvious neurologic deficits developed. Although this may be due to the relatively subtle deficits that would be expected as a result of a lesion in this area, it suggests that more frequent MRI monitoring of patients who receive natalizumab may be warranted. The appearance of lesions, particularly in or abutting the gray matter, should increase clinical suspicion of PML. Monitoring for JC virus viremia may also be useful in such patients. Our case report suggests that some degree of recovery from natalizumab-associated PML is possible.

Supported by a grant (NS43207-03) from the National Institute of Neurological Diseases and Stroke and a Wadsworth Foundation Young Investigators Award (both to Dr. Langer-Gould).

Dr. Langer-Gould reports having received consulting and lecture fees from Biogen Idec; and Dr. Pelletier, consulting fees, lecture fees, and grant support from Biogen Idec.

We are indebted to Kristin Cobb and Michael K. Gould for helpful reviews of the manuscript, to Caroline Ryschkewitsch and Eugene Major for determining the JC virus titers to Mary Owen, Heather Hinds, Keith K. Abe, and Jeffrey H. Gertsch for their outstanding clinical care of the patient, and to the patient and his family for allowing us to report his story.

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Exhibit 4

Combination Therapy with Glatiramer Acetate (Copolymer-1) and a Type I Interferon (IFN-α) Does Not Improve Experimental Autoimmune Encephalomyelitis

Staley A. Brod, MD, J. William Lindsey, MD, and Jerry S. Wolinsky, MD

We sought to determine whether combinations of glatiramer acetate and parenteral or ingested type I interferon were synergistic in experimental autoimmune encephalomyelitis. Glatiramer acetate, subcutaneous murine interferon-α, or ingested murine interferon-α individually improved clinical scores. In contrast, glatiramer acetate in conjunction with either subcutaneous or ingested interferon-α did not improve clinical scores compared with control. These data suggest that clinical trials designed to test a possible synergistic effect of glatiramer acetate and type I interferon in humans should be designed to detect possible adverse effects of this combination of immunomodulatory agents.

Brod SA, Lindsey W, Wolinsky JS. Combination therapy with glatiramer acetate (copolymer-1) and a type 1 interferon (IFN-α) does not improve experimental autoimmune encephalomyelitis. Ann Neurol 2000;47:127–131

Parenteral type I interferon (IFN-α2 [Roferon], IFNβ-1a [Avonex/Rebif], and IFNβ-1b [Betaseron]) and glatiramer acetate (copolymer-1 [Copaxone]) decrease the number of clinical attacks in relapsing-remitting multiple sclerosis (MS) by 17 to 32%.[1–5] IFNα-2a treatment results in fewer new magnetic resonance imaging lesions during the treatment period.[6] Because each therapy alone has a partial therapeutic benefit, a clinical trial to determine if combined use of a parenteral IFNβ-1a (Avonex) and glatiramer acetate is additive or synergistic is underway.[7] Experimental autoimmune encephalomyelitis (EAE) is a T-cell mediated inflammatory autoimmune process of the central nervous system (CNS) which resembles the human demyelinating disease MS in some aspects and provides a model to assess the therapeutic interventions that may influence its course.[8] We have demonstrated that ingested IFN-α can modify biological responses in chronic relapsing EAE in SJL/J mice and acute EAE in Lewis rats.[9] Ingested type 1 interferon is more effective than parenteral (subcutaneous) IFN-α in the suppression of attacks in chronic relapsing EAE.[10] The EAE model may provide insight into the clinical potential of glatiramer acetate and type I interferon combinations and aid in the design of clinical trials. Therefore, we determined whether combinations of glatiramer acetate and parenteral or ingested type I interferon are synergistic in EAE.

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Received Jul 9, 1999, and in revised form Aug 23. Accepted for publication Aug 27, 1999.

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Experimental Methods

In the initial experiments to establish the efficacy of optimal dosing of glatiramer acetate for use in combined intervention experiments, SJL/J mice (n = 8–12 per group) received either 10, 5, or 1 mg of glatiramer acetate (Teva Pharmaceutical Industries, Israel) in incomplete Freund's adjuvant (IFA), and a control group received a mock injection of saline in IFA 3 weeks preceding inoculation (day 0). On day 0, all mice were inoculated with 0.2 mg of encephalitogen (lyophilized mouse spinal cord homogenate [MSCH]) in complete Freund's adjuvant (CFA) and scored by a blinded observer for 21 to 28 days after inoculation. This is the typical prevention experimental model to evaluate glatiramer acetate batch efficacy.[11]

In the second set of three separate experiments, CSJL/F1 or SJL/J mice were divided into two different groups of 24 mice each. The first group received a suboptimal but therapeutic single dose of 1 mg of glatiramer acetate in IFA, and the other group received a mock injection of saline in IFA 3 weeks preceding inoculation (day 0). A suboptimal glatiramer acetate dose was used to enable the detection of any potential additive or synergistic effect of parenteral or ingested type 1 interferon without resorting to excessive numbers of experimental mice to demonstrate such an effect. Beginning at day −7 through and including day +14, 8 mice in each group received either 100 IU of murine IFN-α subcutaneously (Cytimmune mouse IFN-α, 4×10^5 IRU/ml; Lee Biomolecular Research, San Diego, CA) or 10 IU of murine IFN-α by gavage. IFN-α doses were chosen to provide optimal protection in this model. On day 0, all mice were inoculated with 0.2 mg of encephalitogen MSCH, proteolipid protein (PLP) 139–151 peptide, or PLP 178–191 peptide in CFA and were scored by a blinded observer for at least 17 days after inoculation. Spleen cells were cultured with Con A (2.5 mg/ml), *Mycobacterium tuberculosis*, PLP 139–151 at 2 to 20 μg/ml, glatiramer acetate at 3 to 30 μg/ml at 2×10^5 cells per well in 96 round-bottom well

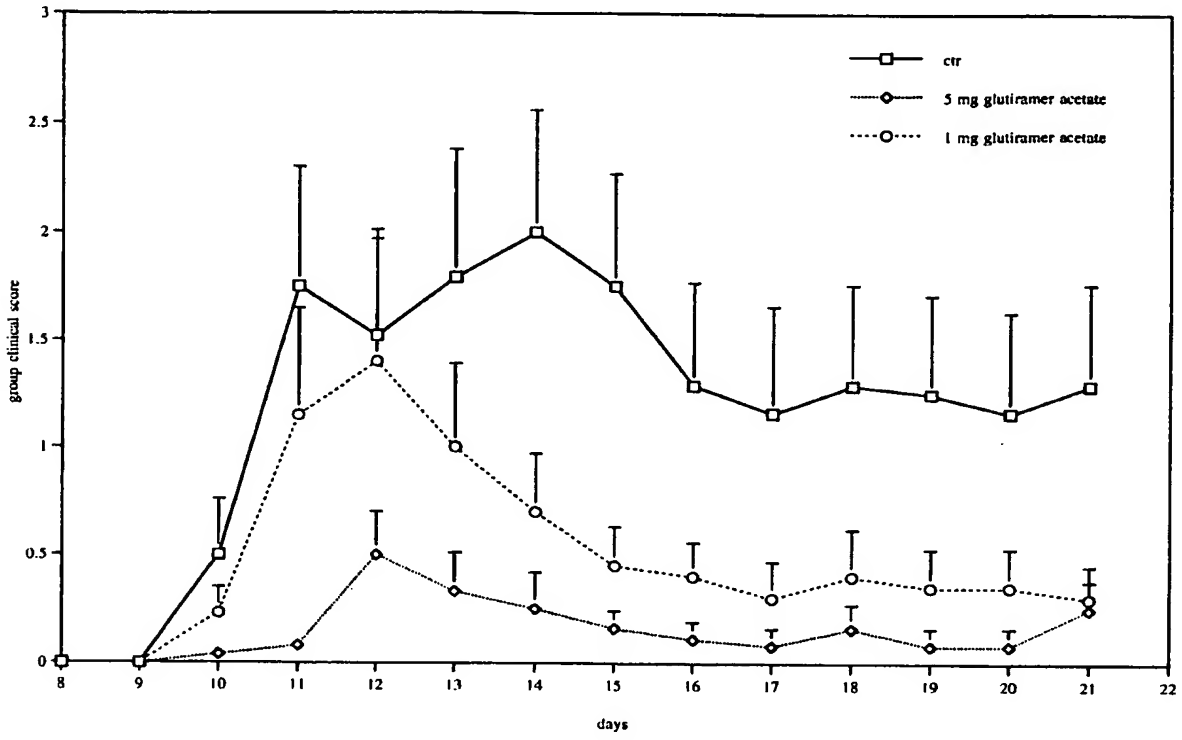


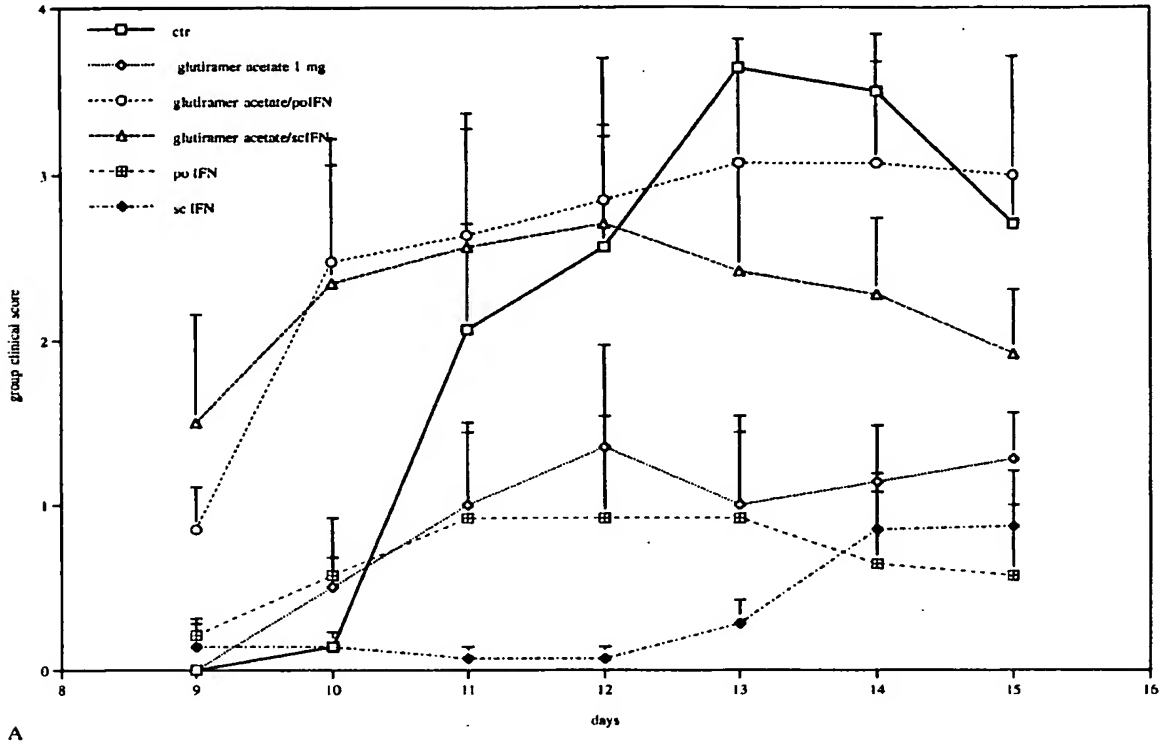
Fig 1. One milligram of glatiramer acetate acts suboptimally in protection against experimental autoimmune encephalomyelitis. SJL/J mice (n = 8 per group) received either 5 or 1 mg of glatiramer acetate in incomplete Freund's adjuvant (IFA), and the control group received a mock injection of saline in IFA 3 weeks preceding inoculation (day 0) with lyophilized mouse spinal cord homogenate (MSCH) in complete Freund's adjuvant (CFA). On day 0, all mice were inoculated with 0.2 mg of MSCH in CFA and were scored by a blinded observer for 21 days after inoculation. Clinical severity of the initial attack was graded as follows by a blinded observer: 0 = no disease, 1 = limp tail or slow righting response, 2 = limp tail and slow righting response, 3 = paraparesis with abnormal gait, 4 = paraplegia but mobile using forelimbs, 5 = inanition, 6 = dead. Mice were scored daily, and a cumulative score was computed by averaging scores for each group of animals ± SEM.

plates in a humidified 5% carbon dioxide and 95% air incubator at 37°C. Supernatants were collected at 48 hours and frozen at −70°C after centrifugation. Interleukins were measured using solid-phase enzyme-linked immunosorbent assay with anti–interleukin-2 (IL-2), anti–IL-4, anti–IL-6, anti–IL-10, anti–tumor necrosis factor-α, anti–transforming growth factor-β, or anti–IFN-γ (PharMingen, San Diego, CA) antibodies as outlined previously.[9]

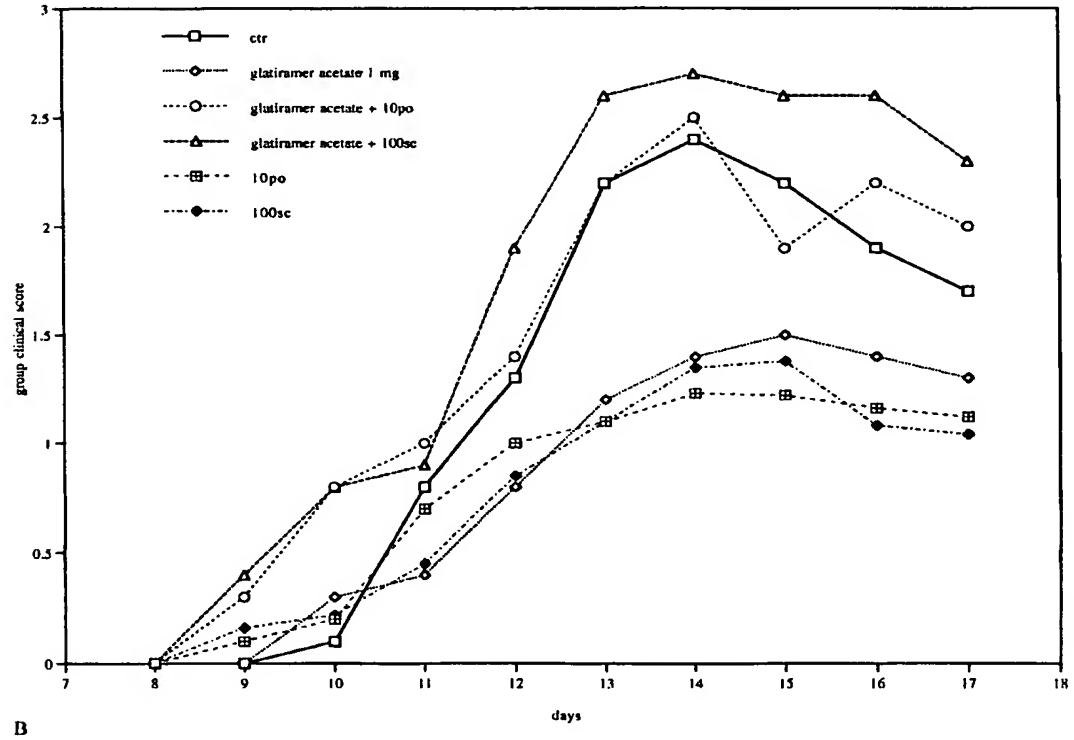
Results and Discussion

We first determined if previously described effective doses of glatiramer acetate were effective in suppressing

Fig 2. Combination therapy with glatiramer acetate and interferon-α (IFN-α) does not improve experimental autoimmune encephalomyelitis. (A) In the first set of experiments, CSJL/J F1 or SJL/J mice were divided into two different groups of 24 mice each. The first group received 1 mg of glatiramer acetate in incomplete Freund's adjuvant (IFA), and the other group received a mock injection of saline in IFA 3 weeks preceding inoculation (day 0). Beginning at day −7 through and including day +14, 8 mice in each group received either 100 IU of murine IFN-α subcutaneously or 10 IU of murine IFN-α by gavage. On day 0, all mice were inoculated with 0.2 mg of encephalitogen (lyophilized mouse spinal cord homogenate [MSCH]) in complete Freund's adjuvant (CFA) and were scored by a blinded observer for at least 15 days after inoculation. (B) In cumulative results from three separate experiments, CSJL/J F1 or SJL/J mice were divided into two different groups of 72 mice each. The first group received 1 mg of glatiramer acetate in IFA, and the other group received a mock injection of saline in IFA 3 weeks preceding inoculation (day 0). Beginning at day −7 through and including day +14, 24 mice in each group received either 100 IU of murine IFN-α subcutaneously or 10 IU of murine IFN-α by gavage. On day 0, all mice were inoculated with 0.2 mg of encephalitogen (MSCH, proteolipid protein [PLP] 139–151 peptide, or PLP 178–191 peptide) in CFA and were scored by a blinded observer for at least 17 days after inoculation.


ctr
glutiramer acetate 1 mg
glutiramer acetate/poIFN
glutiramer acetate/scIFN
po IFN
sc IFN
group clinical score
days

A


ctr
glatiramer acetate 1 mg
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10po
100sc
group clinical score
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B

EAE. We performed an initial set of experiments in which mice received either 10 mg of glatiramer acetate in IFA or a mock injection of saline in IFA 3 weeks preceding inoculation with MSCH (day 0). Mice receiving an optimal dose of glatiramer acetate (group clinical score: 4.3 ± 0.6 [SE]) had much less severe clinical disease compared with the control group (group clinical score: 0.8 ± 0.4 [SE]). Based on these results, sample size projections to determine a 50% or greater additive effect of IFN-α on clinical outcome projected the requirement of 200 mice per study arm. Therefore, to demonstrate any potential additive or synergistic effect and to more readily detect any deleterious effect of parenteral or ingested type 1 interferon used in combination with glatiramer acetate, we sought a suboptimal glatiramer acetate dose. Additional experiments were performed with glatiramer acetate alone in which mice received either 5 or 1 mg of glatiramer acetate in IFA or a mock injection of saline in IFA 3 weeks preceding inoculation with MSCH (day 0). The 5-mg dose still provided good protection against EAE, whereas the 1-mg dose was less effective, particularly early in the course of disease (Fig 1). Therefore, we used the 1-mg glatiramer acetate dose in experiments modeling combined treatment with either parenteral or ingested IFN-α.

Subsequently, we examined the effect of combinations of 1 mg of glatiramer acetate and optimal subcutaneous or ingested IFN-α doses in mice inoculated as described above. In the first set of experiments, control, glatiramer acetate and ingested IFN-α, and glatiramer acetate and subcutaneous IFN-α all showed at least a mean group clinical score of 2.5, whereas the glatiramer acetate, subcutaneous murine IFN-α, and ingested murine IFN-α individually had mean group clinical scores no greater than 1.3 (Fig 2A). In the cumulative data from three separate experiments, clinical disease began on day 9, 2 days before disease onset in the control mice, in the groups using glatiramer acetate in conjunction with either subcutaneous or ingested murine IFN-α (see Fig 2B). The maximal group clinical scores in all groups occurred at day 14 postimmunization. Control mice showed a mean maximal group clinical score equal to 2.4 ± 0.6 (SE). The glatiramer acetate group showed a mean maximal group clinical score equal to 1.53 ± 0.4 (SE), ingested IFN-α of 1.3 ± 0.6 (SE), subcutaneous IFN-α of 1.5 ± 0.6 (SE), glatiramer acetate and ingested IFN-α of 2.6 ± 0.3 (SE), and glatiramer acetate and subcutaneous IFN-α of 2.7 ± 0.2 (SE). Clinical scores were compared between groups by the Mann-Whitney rank sum test at days 12 through 17 postinoculation. Glatiramer acetate, subcutaneous murine IFN-α, and ingested murine IFN-α individually improved clinical scores significantly at days 13 and 14 compared with control ($p < 0.05$). In contrast, glatiramer acetate in conjunction with either subcutaneous or ingested IFN-α did not improve clinical scores on days 12 through 17 compared with control. These experiments demonstrate that a combination of either ingested or subcutaneous murine IFN-α in conjunction with glatiramer acetate abrogates the clinical effectiveness of either therapy. In several experiments, cytokine analysis of antigen- or mitogen-stimulated spleen cell IL-2, IL-4, IL-6, IL-10, tumor necrosis factor-α, transforming growth factor-β, or IFN-γ production demonstrated no significant differences in cytokine production between control mice and groups treated with either IFN-α, glatiramer acetate, or glatiramer acetate and IFN-α (data not shown).

Glatiramer acetate (copolymer-1) is a synthetic amino acid copolymer that inhibits the T-cell response to myelin basic protein, suppresses EAE in many animal species,[12] exhibits cross-reactivity with myelin basic protein, and shows a progressively polarized development toward the Th2 pathway by secretion of IL-4, IL-6, and IL-10. Glatiramer acetate T cells ameliorated EAE induced by two unrelated encephalitogenic epitopes of PLP: p139–151 and p178–191, suggesting bystander suppression as the mechanism of action.[13,14]

IFN-β, an immune modulator with no antigenic specificity, delays the onset and decreases the severity of EAE symptoms, inflammation, and demyelination.[15,16] The beneficial effect of IFN-β treatment in MS patients may be in part a result of its ability to decrease metalloproteinase-9 activity of T cells, which allows migration of activated T lymphocytes through the cerebral endothelium, leading to a reduction of CNS T-lymphocyte infiltration.[17]

In the intact animal model, the effect of parenteral or ingested IFN-α on metalloproteinase-9 could result in ablation of the therapeutic efficacy of glatiramer acetate by inhibition of glatiramer acetate Th2 T-cell lymphocyte infiltration into the CNS. It is proposed that these cells may need to gain access to the CNS to downregulate Th1-mediated tissue destruction. Glatiramer acetate may inhibit the activity of type I interferon by stimulating the production of IL-10 and IL-4 from responsive Th2 cells. IL-10 can directly inhibit STAT-dependent early-response gene expression induced by IFN-α by suppressing the tyrosine phosphorylation of STAT1, an important intermediary in type I interferon signal transduction.[18] IL-4 inhibits interferon-induced RNA levels by reducing the transcriptional activator, interferon-stimulated gene factor 3.[19] As a result, in the animals undergoing combined treatment, the benefits of either drug used alone are lost.

It is possible that the optimal IFN-α dose by mouth (10 IU) or by injection (100 IU) is not the optimal dose in conjunction with glatiramer acetate. We used a suboptimal dose of glatiramer acetate to determine the synergistic or deleterious effect of ingested or parenteral

IFN-α. The optimal dose of glatiramer acetate (10 mg) in conjunction with lower IFN-α doses may have demonstrated an additive or synergistic effect. Similarly, different timing of the introduction of the two drugs or use in a model of relapsing or progressive EAE after initial disease induction might result in a different outcome. Our inability to detect differences in proinflammatory of anti-inflammatory cytokine production between the glatiramer acetate, ingested and parenteral IFN-α groups, and control and combination groups may reflect the performance of assays at days 17 and 18, after peak group clinical disease differences had passed. Clinical trials designed to test a possible additive or synergistic effect of glatiramer acetate (Copaxone) and IFN-β (Avonex) in man should be designed to detect possible adverse effects of this combination of immunomodulatory agents.

This study was supported by a grant from Teva Pharmaceutical Industries, Israel.

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Exhibit 5

Multiple Sclerosis 2003; 9: 28–31
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Effects of combination therapy of beta-interferon 1a and prednisone on serum immunologic markers in patients with multiple sclerosis

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Beta-interferon (beta-IFN) has a proven treatment effect on relapsing–remitting multiple sclerosis (MS), presumably through its regulatory properties on T-cell activation and cytokine production. This paper examines whether combination therapy of beta-IFN with prednisone would enhance immunoregulatory effects of beta-IFN by measuring serum levels of selected proinflammatory cytokines and soluble T-cell activation markers associated with MS. The selected markers were analyzed in MS patients treated with beta-IFN alone (n = 22) and beta-IFN combined with a low daily dose of prednisone (n = 33), as compared with those in 27 healthy controls at baseline and at a three-month interval for one year. The study confirmed that beta-IFN treatment inhibited serum levels of tumor necrosis factor-alpha (TNFα) and intracellular adhesion molecule-1 (ICAM-1) in patients with MS. However, combination therapy did not significantly enhance the inhibitory effect of beta-IFN treatment on the production of TNFα, interleukin (IL)-12, IL-2R, and ICAM-1, while the addition of prednisone antagonized the effect of beta-IFN on up-regulation of IL-10 and soluble CD95. No difference in the occurrence of binding antibodies to beta-IFN was found between the two treatment groups. The findings are important for the understanding of the role of combination therapy in the treatment of MS.
Multiple Sclerosis *(2003)* 9, 28–31

Key words: beta-interferon; cytokines; multiple sclerosis; T cells

Introduction

Multiple sclerosis (MS) is a common demyelinating and inflammatory disease of the central nervous system (CNS) with a presumed autoimmune etiology.[1] Despite the CNS location of MS pathology, the inflammatory/autoimmune processes in MS are not entirely confined to the CNS and are associated with T-cell activation and the production of proinflammatory cytokines [e.g., tumor necrosis factor-alpha (TNF_a) and interleukin (IL)-12], adhesion molecules [e.g., intracellular adhesion molecule-1 (ICAM-1)], and soluble T-cell activation markers (e.g., IL-2 receptors), which reflects systemic immune abnormality.[2] Serum levels of some immunologic markers are found to correlate with clinical exacerbation in MS patients, and may be of value in our attempt to monitor the disease processes and the treatment effects in MS.[3,4] This study was undertaken to evaluate whether an immunosuppressive drug, such as prednisone, used at a low oral dose may have add-on or synergistic properties when combined with beta-interferon (beta-IFN), an immunomodulatory drug, and whether the immunoregulatory effects of the drugs in combination could be measured sensitively by detecting the changes in selected serum immunologic markers as they are potentially associated with MS.

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Received 11 February 2002; accepted 18 June 2002

Materials and methods

Patients and specimens

Fifty-five clinically definite MS patients with a relapsing–remitting or secondary progressive disease were included in the study. The clinical characteristics of the patients are shown in Table 1. These patients were not treated with any immunomodulatory or immunosuppressive drugs at least three months prior to the study. Patients were assigned to receive either beta-IFN-1a (Avonex) monotherapy at 30 mg by weekly intramuscular (im) injections ($n = 22$) or beta-IFN-1a combined with prednisone at a daily oral dose of 15 mg ($n = 33$). In this study, the dose of prednisone was selected based on the clinical experience at the Indiana Center for Multiple Sclerosis and Neuroimmunopathologic Disorders. Serum specimens were collected before the initiation of the treatments and three, six, nine, and 12 months after the treatments. Patients in relapse were not excluded from the assays throughout the study. Control serum specimens were obtained in parallel from a group of healthy volunteers ($n = 27$) at the same schedule. The study was approved by the Institutional Review Board for Human Subjects Study.

10.1191/1352458503ms865oa

Table 1 Demographic data and clinical characteristics of patients and controls

Group	*n*	*RR/SP*	*Treatment*	*Age*	*Sex*	*MS duration (year)*	*Pre-EDSS*	*Post-EDSS*
MS	22	14/8	Beta-IFN-1a	40.8± 9	15F/7M	8.7± 7.4	2.4± 1.6	2.3± 1.7
MS	33	22/11	Beta-IFN-1a, prednisone	38.5± 7.3	22F/11M	8.3± 5.9	2.5± 1.5	2.5± 1.6
Controls	27	–	None	33.7± 8.0	19F/8M	–	–	–

RR, relapsing–remitting MS; SP, secondary progressive MS; EDSS, Expanded Disability Scale Score; Controls, healthy individuals; Pre-EDSS, baseline EDSS; Post-EDSS, EDSS at 12 months after treatment

Measurements of serum cytokines and soluble factors

Serum specimens were assayed for concentrations of TNF_a, IL-12, IL-2R, IL-10, CD95, and ICAM-1 using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. Briefly, microtiter plates precoated with capturing monoclonal antibodies to the selected markers were incubated with 10% (w/v) fetal bovine serum (FBS) to saturate nonspecific binding sites. Biotinylated detecting antibodies were added subsequently to each well and incubated at room temperature for two hours. After washing, avidin-conjugated horseradish peroxidase was added and plates were incubated for one hour. 3,3′,5,5′-Tetramethylbenzidine was used as a substrate for color development. Optical density was measured at 450 nm by using an ELISA reader and the concentrations of the selected markers were quantified by Microplate computer software (Bio-Rad, Hercules, CA, USA).

Results and discussion

As shown in Figure 1, pretreatment serum levels of TNF_a, IL-12, IL-2R, and ICAM-1 were significantly elevated in MS patients, even though there were considerable discrepancies in the levels of IL-2R and IL-12 between the two groups of MS patients. The levels of IL-10 and soluble CD95 did not differ significantly between the MS group and the control group. The study revealed that treatment with beta-IFN-1a alone significantly reduced levels of TNF_a (300 pg/mL to 105 pg/mL at month 12, $P < 0.001$) and ICAM-1 (318 ng/mL to 217 ng/mL at month 12, $P < 0.004$) but not IL-2R and IL-12 in this group of MS patients. In contrast, the production of IL-10 and soluble CD95 was considerably increased after the treatment with beta-IFN-1a alone. IL-10 was increased from 381 pg/mL to 456 pg/mL at month 9 ($P < 0.003$) and the levels of soluble CD95 were elevated from 2.94 ng/mL to 6.23 ng/mL ($P < 0.001$). The observed changes in the serum levels of the selected cytokines and soluble markers in MS patients treated with beta-IFN are consistent with the previous studies.[5–7]

In the MS group that received the combination therapy, the addition of prednisone did not significantly alter the serum levels of TNF_a, IL-2R, IL-12, and ICAM-1 at the end of the 12-month study, even though the combination therapy seemed to enhance the inhibition of ICAM-1 and IL-12 in the first three months as compared with that seen in the MS group treated with beta-IFN-1a alone. In contrast, it was demonstrated for the first time that the addition of prednisone at the given dose in the combination therapy appeared to antagonize the up-regulatory effect of beta-IFN-1a on IL-10 and soluble CD95 by suppressing both serum markers. In general, the changes in some cytokines/markers, such as sCD95 and IL-10, are more clear than those of the other selected markers (e.g., IL-12, TNF), which is also reflected by statistical analysis. This could be due to the relative effects of the treatment on the serum levels of certain cytokines/markers. Further experiments revealed the same occurrence of serum binding antibodies to beta-IFN in both treatment groups (2/22 versus 3/33) at six months and 12 months after treatment, suggesting that the addition of prednisone had no effect on the production of binding antibodies induced by the beta-IFN treatment (data not shown).

It has been proposed that the regulatory effects of beta-IFN may be enhanced by combining with other immunomodulating or immunosuppressive agents, such as prednisone, which is known to inhibit inflammatory responses of predominantly T-cell populations. This study suggests that combination therapy seems to add some synergistic effects with beta-IFN-1a on the production of ICAM-1 and IL-12 during the first three months of treatment and makes no difference at the end of the 12-month treatment. More importantly, the combination of beta-IFN and prednisone appears to produce multiple effects on the immune system. The findings described here suggest that the addition of prednisone in combination therapy antagonizes the effect of beta-IFN-1a on the up-regulation of IL-10, a potent anti-inflammatory cytokine mainly produced by Th2 cells. As IL-10 is known to suppress experimental autoimmune excepahlomyelitis, an animal model for MS,[8] and is associated with clinical benefit induced by beta-IFN treatment in MS,[7] there is a likelihood that suppression of IL-10 production by the combination therapy might compromise the treatment effect of beta-IFN. Furthermore, the combination therapy completely abolishes the ability of beta-IFN-1a to promote the production/release of soluble CD95. Soluble CD95 is a natural inhibitor for Fas/FasL-mediated T-cell apoptosis, one of the important mechanism of T-cell regulation, and its serum level has been found to be elevated in MS patients.[9] Unlike IL-10, the significance of the reduction in serum-soluble CD95 in relationship to the treatment effect in MS is not clear at the present time.

In this pilot study, potential adverse reactions of the combination therapy were evaluated systemically. No significant side effects were associated with the combina-

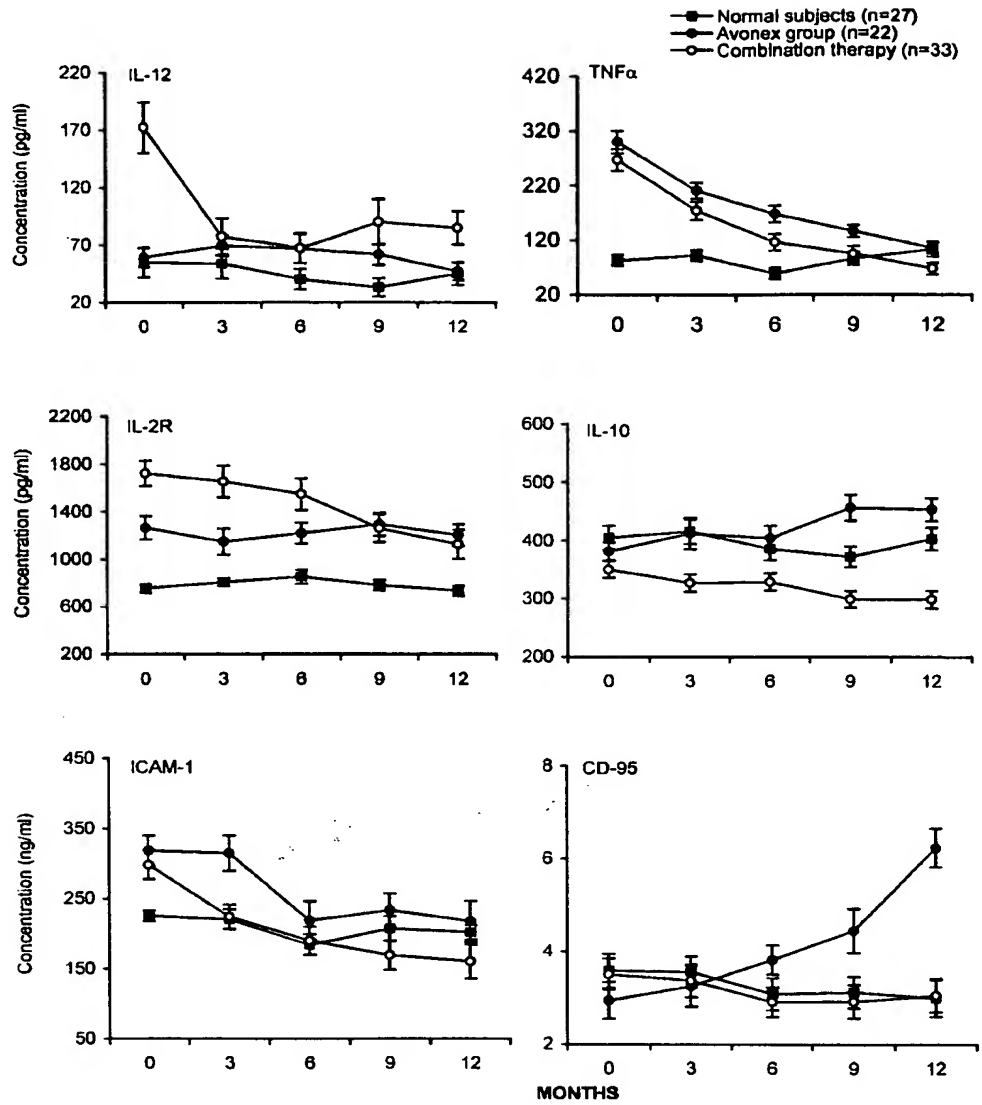

Figure 1 *Serum levels of the selected immunologic markers in relation to treatment with beta-IFN alone or in combination with prednisone over a 12-month period. Serum specimens were assayed for the selected immunologic markers by ELISA and the concentrations were determined using standard curves included in all assays. Data are presented as concentrations (ng/mL or pg/mL)± SEM. Statistical analysis was performed using Student's t-test for normally distributed variables and the Mann–Whitney rank-sum test for non-normally distributed variables.*

tion therapy except the 'flu-like' symptoms that were also seen in patients treated with beta-IFN-1a. Three of 22 patients in the beta-IFN-1a group and five of 33 patients in the combination treatment group had a single relapse during the study. No conclusion could be drawn from this study as to whether the combination therapy is more clinically beneficial due to the small number of patients and the limited clinical assessment. Further investigations with a large sample size and in controlled clinical trials are needed to address the treatment efficacy issues as well as to confirm whether the assessment of serum levels of relevant immunologic markers associated with MS is of value in the evaluation of the treatment effect of potential therapeutic agents.

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Exhibit 6

Guidance for Industry

In Vivo Drug Metabolism/Drug Interaction Studies — Study Design, Data Analysis, and Recommendations for Dosing and Labeling

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologics Evaluation and Research (CBER)
November 1999
Clin/Pharm

Guidance for Industry

In Vivo Drug Metabolism/Drug Interaction Studies — Study Design, Data Analysis, and Recommendations for Dosing and Labeling

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November 1999
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TABLE OF CONTENTS

Guidance for Industry[1]

In Vivo Drug Metabolism/Drug Interaction Studies — Study Design, Data Analysis, and Recommendations for Dosing and Labeling

I. INTRODUCTION

This guidance provides recommendations to sponsors of new drug applications (NDAs) and biologics license applications (BLAs) for therapeutic biologics (hereafter drugs) who intend to perform in vivo drug metabolism and metabolic drug-drug interaction studies. The guidance reflects the Agency's current view that the metabolism of an investigational new drug should be defined during drug development and that its interactions with other drugs should be explored as part of an adequate assessment of its safety and effectiveness. For metabolic drug-drug interactions, the approaches considered in the guidance are offered with the understanding that whether a particular study should be performed will vary, depending on the drug in development and its intended clinical use. Furthermore, not every drug-drug interaction is metabolism-based, but may arise from changes in pharmacokinetics caused by absorption, tissue and/or plasma binding, distribution, and excretion interactions. Drug interactions related to transporters are being documented with increasing frequency and may be addressed more fully in future guidances. Although less well studied, drug-drug interactions may alter pharmacokinetic/pharmacodynamic (PK/PD) relationships. These important areas are not considered in detail in this guidance.

Previous guidance from FDA on the use of in vitro approaches to study drug metabolism and metabolic drug-drug interactions is available in a guidance document entitled *Drug Metabolism/Drug Interaction Studies in the Drug Development Process: Studies In Vitro* (April 1997). The present guidance should be viewed as a companion to this earlier guidance. Discussion of metabolic and other types of drug-drug interactions is also provided in other guidances, including the International Conference on Harmonisation (ICH) *E8 General Considerations for Clinical Trials* (December 1997), *E7 Studies in Support of Special Populations: Geriatrics* (August 1994), and *E3 Structure and Content of Clinical Study Reports* (July 1996), and the Agency guidances *Studying Drugs Likely to be Used in the Elderly* (November 1989) and *Study and Evaluation of Gender Differences in the Clinical Evaluation of Drugs* (July 1993).

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[1] This guidance has been prepared by the In Vivo Metabolic Drug-Drug Interaction Working Group in the Clinical Pharmacology Section of the Medical Policy Coordinating Committee in the Center for Drug Evaluation and Research, with input from the Center for Biologics Evaluation and Research, at the Food and Drug Administration. This guidance document represents the Agency's current thinking on the subject of in vivo drug metabolism and metabolic drug-drug interactions. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. An alternative approach may be used if such approach satisfies the requirements of the applicable statutes, regulations, or both.

II. BACKGROUND

A. Metabolism

The desirable and undesirable effects of a drug arising from its concentrations at the sites of action are usually related either to the amount administered (dose) or to the resulting blood concentrations, which are affected by its absorption, distribution, metabolism and/or excretion. Elimination of a drug or its metabolites occurs either by metabolism, usually by the liver, or by excretion, usually by the kidneys and liver. In addition, protein therapeutics may be eliminated via a specific interaction with cell surface receptors, followed by internalization and lysosomal degradation within the target cell. Hepatic elimination occurs primarily by the cytochrome P450 family of enzymes located in the hepatic endoplasmic reticulum but may also occur by non-P450 enzyme systems, such as N-acetyl and glucuronosyl transferases. P450 enzyme systems located in gut mucosa can also significantly affect the amount of drug absorbed into the systemic circulation.[2] Many factors can alter hepatic and intestinal drug metabolism, including the presence or absence of disease and/or concomitant medications. While most of these factors are usually relatively stable over time, concomitant medications can alter metabolic routes of absorption and elimination abruptly and are of particular concern. The influence of concomitant medications on hepatic and intestinal metabolism becomes more complicated when a drug, including a prodrug, is metabolized to one or more active metabolites. In this case, the safety and efficacy of the drug/prodrug are determined not only by exposure to the parent drug but by exposure to the active metabolites, which in turn is related to their formation, distribution, and elimination.

B. Metabolic Drug-Drug Interactions

Many metabolic routes of elimination, including most of those occurring via the P450 family of enzymes, can be inhibited, activated, or induced by concomitant drug treatment. Observed changes arising from metabolic drug-drug interactions can be substantial — an order of magnitude or more decrease or increase in the blood and tissue concentrations of a drug or metabolite — and can include formation of toxic metabolites or increased exposure to a toxic parent compound. Examples of substantially changed exposure associated with administration of another drug include (1) increased levels of terfenadine, cisapride, or astemizole with ketoconazole or erythromycin (inhibition of CYP3A4); (2) increased levels of simvastatin and its acid metabolite with mibefradil or itraconazole (inhibition of CYP3A4); (3) increased levels

[2] No distinction is made in this document between the effects of concomitant drugs and/or alterations in metabolism on gastrointestinal absorption and hepatic elimination, although the pharmacokinetic effects of the two may be different.

of desipramine with fluoxetine, paroxetine, or quinidine (inhibition of CYP2D6); and (4) decreased carbamazepine levels with rifampin (induction of CYP3A4). These large changes in exposure can alter the safety and efficacy profile of a drug and/or its active metabolites in important ways. This is most obvious and expected for a drug with a narrow therapeutic range (NTR), but is also possible for non-NTR drugs as well (e.g., HMG CoA reductase inhibitors). Depending on the extent and consequence of the interaction, the fact that a drug's metabolism can be significantly inhibited by other drugs and that the drug itself can inhibit the metabolism of other drugs can require important changes in either its dose or the doses of drugs with which it interacts, that is, on its labeled conditions of use. Rarely, metabolic drug-drug interactions may affect the ability of a drug to be safely marketed.

The following general concepts underlie the recommendations in this guidance:

- Adequate assessment of the safety and effectiveness of a drug includes a description of its metabolism and the contribution of metabolism to overall elimination.

- Metabolic drug-drug interaction studies should explore whether an investigational agent is likely to significantly affect the metabolic elimination of drugs already in the marketplace and, conversely, whether drugs in the marketplace are likely to affect the metabolic elimination of the investigational drug.

- Even drugs that are not substantially metabolized can have important effects on the metabolism of concomitant drugs. For this reason, metabolic drug-drug interactions should be explored, even for an investigational compound that is not eliminated significantly by metabolism.

- In some cases, metabolic drug-drug interaction studies cannot be informative unless metabolites and prodrugs have been identified and their pharmacological properties described.

- Identifying metabolic differences in patient groups based on genetic polymorphism, or on other readily identifiable factors, such as age, race, and gender, can aid in interpreting results.

- The impact of an investigational or approved interacting drug can be either to inhibit or induce metabolism.

- A specific objective of metabolic drug-drug interaction studies is to determine whether the interaction is sufficiently large to necessitate a dosage adjustment of the drug itself or the drugs it might be used with, or whether the interaction would require additional therapeutic monitoring.

- In some instances, understanding how to adjust dosage in the presence of an interacting drug, or how to avoid interactions, may allow marketing of a drug that would otherwise have been associated with an unacceptable level of toxicity. Sometimes a drug interaction may be used intentionally to increase levels or reduce elimination of another drug. Rarely, the degree of interaction caused by a drug, or the degree to which other drugs alter its metabolism, may be such that it cannot be marketed safely.

- The blood or plasma concentrations of the parent drug and/or its active metabolites (systemic exposure) may provide an important link between drug dose (exposure) and desirable and/or undesirable drug effects. For this reason, the development of sensitive and specific assays for a drug and its key metabolites is critical to the study of metabolism and drug-drug interactions.

- For drugs whose presystemic or systemic clearance occurs primarily by metabolism, differences arising from various sources, including administration of another drug, are an important source of inter-individual and intra-individual variability.

- Unlike relatively fixed influences on metabolism, such as hepatic function or genetic characteristics, metabolic drug-drug interactions can lead to abrupt changes in exposure. Depending on the nature of the drugs, these effects could potentially occur when a drug is initially administered, when it has been titrated to a stable dose, or when an interacting drug is discontinued. Interactions can occur after even a single concomitant dose of an inhibitor.

- The effects of an investigational drug on the metabolism of other drugs and the effects of other drugs on an investigational drug's metabolism should be assessed relatively early in drug development so that the clinical implications of interactions can be assessed as fully as possible in later clinical studies.

III. GENERAL STRATEGIES

To the extent possible, drug development should follow a sequence where early in vitro and in vivo investigations can either fully address a question of interest or provide information to guide further studies. Optimally, a sequence of studies should be planned, moving from in vitro studies, to early exploratory studies, to later more definitive studies, employing special study designs and methodology where necessary and appropriate. In many cases, negative findings from early in vitro and early clinical studies can eliminate the need for later clinical investigations. Early investigations should explore whether a drug is eliminated primarily by excretion or metabolism, with identification of the principal metabolic routes in the latter case. Using suitable in vitro probes and careful selection of interacting drugs for early in vivo studies, the potential for drug-drug interactions can be studied early in the development process, with further study of observed interactions assessed later in the process, as needed. In certain cases and with careful study designs and planning, these early studies may also provide information about dose, concentration, and response relationships in the general population, subpopulations, and individuals, which can be useful in interpreting the consequences of a metabolic drug-drug interaction.

A. In Vitro Studies

A complete understanding of the relationship between in vitro findings and in vivo results of metabolism/drug-drug interaction studies is still emerging. Nonetheless, in vitro studies can frequently serve as an adequate screening mechanism to rule out the importance of a metabolic pathway and drug-drug interactions that occur via this pathway so that subsequent in vivo testing is unnecessary. This opportunity should be based on appropriately validated experimental methods and rational selection of substrate/interacting drug concentrations. For example, if suitable in vitro studies indicate that CYP2D6 or 3A4 enzyme systems do not metabolize an investigational drug, then clinical studies to identify the impact of the CYP2D6 slow metabolizer phenotype or to study the effect of CYP2D6 inhibitors or CYP3A4 inhibitors/inducers on the elimination of the investigational drug will not be needed. Similarly, if in vitro studies indicate that an investigational drug does not inhibit CYP2D6 or 3A4 metabolism, then corresponding in vivo drug-drug interaction studies of the investigational drug and concomitant medications eliminated by these pathways are not needed.

In contrast, when positive findings arise in in vitro metabolic and/or drug-drug interaction studies, clinical studies are recommended because of the limited ability at present of in vitro findings to give a good quantitative estimate of the clinical importance of a metabolic pathway or interaction. Further evaluation of the utility of parameters such as the ratio between the concentration of drug and the Ki (inhibition constant) for the interaction may lead to continued improvements in the ability of in vitro studies to predict in vivo results, but the overall experience to date is not large enough to allow reliable conclusions. Although in vitro studies can assess the presence or absence of inhibition, they have limited capability to identify

induction. For this reason, in vivo studies remain the primary source of information about induction of metabolic pathways caused by concomitant medications.

B. Specific In Vivo Clinical Investigations

Appropriately designed pharmacokinetic studies, usually performed in the early phases of drug development, can provide important information about metabolic routes of elimination, their contribution to overall elimination, and metabolic drug-drug interactions. Together with information from in vitro studies, these investigations can be a primary basis of labeling statements and can often help avoid the need for further investigations. Further recommendations about these types of studies appear in section IV of this guidance.

C. Population Pharmacokinetic Screens

Population pharmacokinetic analyses of data obtained from blood samples collected infrequently (sparse sampling) in clinical studies conducted in the later phase of clinical drug development can be valuable in characterizing the clinical impact of known or newly identified interactions, and in making recommendations for dosage modifications. It may be possible that analysis or skillful examination of such data could detect unsuspected drug-drug interactions. Population pharmacokinetic data can also provide further evidence of the absence of a pharmacokinetic drug-drug interaction when this is suggested by in vitro drug-drug interaction studies. The power of a sparse sampling strategy to detect drug-drug interactions is not yet well established, however, and it is unlikely that population analysis can be used to prove the absence of an interaction that is strongly suggested by information arising from in vitro or in vivo studies specifically designed to assess a drug-drug interaction. To be optimally informative, population pharmacokinetic studies should have carefully designed study procedures and sample collections. A guidance for industry entitled *Population Pharmacokinetics* was published in February 1999.

IV. DESIGN OF IN VIVO METABOLIC DRUG-DRUG INTERACTION STUDIES

If in vitro studies and other information suggest a need for in vivo metabolic drug-drug interaction studies, the following general issues and approaches should be considered. In the following discussion, the term *substrate* (S) is used to indicate the drug studied to determine if its exposure is changed by another drug, which is termed the *interacting drug* (I). Depending on the study objectives, the substrate and the interacting drug may be the investigational agents or approved products.

A. Study Design

In vivo metabolic drug-drug interaction studies generally are designed to compare substrate levels with and without the interacting drug. Because a specific study may consider a number of questions and clinical objectives, no one correct study design for studying drug-drug interactions can be defined. A study can use a randomized crossover (e.g., S followed by S+I, S+I followed by S), a one-sequence crossover (e.g., S always followed by S+I or the reverse), or a parallel design (S in one group of subjects and S+I in another). The following possible dosing regimen combinations for a substrate and interacting drug may also be used: single dose/single dose, single dose/multiple dose, multiple dose/single dose, and multiple dose/multiple dose. The selection of one of these or another study design depends on a number of factors for both the substrate and interacting drug, including (1) acute or chronic use of the substrate and/or interacting drug; (2) safety considerations, including whether a drug is likely to be an NTR (narrow therapeutic range) or non-NTR drug; (3) pharmacokinetic and pharmacodynamic characteristics of the substrate and interacting drugs; and (4) the need to assess induction as well as inhibition. The inhibiting/inducing drugs and the substrates should be dosed so that the exposure of both drugs are relevant to their clinical use. The following considerations may be useful:

- Pharmacokinetic measures and/or parameters may be used to indicate clinically important routes of metabolism and drug-drug interactions. Subsequent interpretation of findings from these studies will be aided by a good understanding of dose/concentration and concentration/response relationships for both desirable and undesirable drug effects in the general population, in subpopulations, and within individuals. In certain instances, reliance on endpoints other than pharmacokinetic measures/parameters may be useful.

- When both substrate and interacting drug are likely to be given chronically over an extended period of time, administration of the substrate to steady state with collection of blood samples over one or more dosing intervals could be followed by multiple dose administration of the interacting drug, again with collection of blood for measurement of both the substrate and the interacting drug (as feasible) over the same intervals. This is an example of a one-sequence crossover design.

- The time at steady state before collection of endpoint observations depends on whether inhibition or induction is to be studied. Inducers can take several days or longer to exert their effects, while inhibitors generally exert their effects more rapidly. For this reason, a more extended period of time after attainment of steady state for the substrate and interacting drug may be necessary if induction is to be assessed.

- When attainment of steady state is important and either the substrate or interacting drugs and/or their metabolites exhibit long half-lives, special approaches may be useful. These include use of a loading dose to achieve steady state conditions more rapidly and

selection of a one-sequence crossover or a parallel design, rather than a randomized crossover study design.

- When a substrate and/or an interacting drug is to be studied at steady state, documentation that near steady state has been attained is important both for each drug and its metabolites of interest. This documentation can be accomplished by sampling over several days prior to the periods when samples are collected. This is important for both metabolites and the parent drug, particularly when the half-life of the metabolite is longer than the parent, and is especially important if both parent drug and metabolites are metabolic inhibitors or inducers.

- Studies can usually be open label (unblinded), unless pharmacodynamic endpoints (e.g., adverse events that are subject to bias) are part of the assessment of the interaction.

- For a rapidly reversible inhibitor, administration of the interacting drug either just before or simultaneously with the substrate on the test day might be the appropriate design to increase sensitivity.

- If the drug interaction effects are to be assessed for both agents in a combination regimen, the assessment can be done in two separate studies. If the pharmacokinetic and pharmacodynamic characteristics of the drugs make it feasible, the dual assessment can be done in a single study. Some design options are randomized three-period crossover, parallel group, and one-sequence crossover.

B. Study Population

Clinical drug-drug interaction studies may generally be performed using healthy volunteers or volunteers drawn from the general population, on the assumption that findings in this population should predict findings in the patient population for which the drug is intended. Safety considerations, however, may preclude the use of healthy subjects. In certain circumstances, subjects drawn from the general population and/or patients for whom the investigational drug is intended offer certain advantages, including the opportunity to study pharmacodynamic endpoints not present in healthy subjects and reduced reliance on extrapolation of findings from healthy subjects. In either patient or healthy/general population subject studies, performance of phenotype or genotype determinations to identify genetically determined metabolic polymorphisms is often important in evaluating effects on enzymes with polymorphisms, notably CYP2D6 and CYP2C19.

C. Choice of Substrate and Interacting Drugs

1. Substrates for an Investigational Drug

In contrast to earlier approaches that focused mainly on a specific group of approved drugs (digoxin, hydrochlorothiazide) where coadministration was likely or the clinical consequences of an interaction were of concern, improved understanding of the metabolic basis of drug-drug interactions enables more general approaches to and conclusions from specific drug-drug interaction studies. In studying an investigational drug as the interacting drug, the choice of substrates (approved drugs) for initial in vivo studies depends on the P450 enzymes affected by the interacting drug. In testing inhibition, the substrate selected should generally be one whose pharmacokinetics is markedly altered by coadministration of known specific inhibitors of the enzyme systems (i.e., a very sensitive substrate should be chosen) to assess the impact of the interacting investigational drug. Examples of substrates include, but are not limited to, (1) midazolam, buspirone, felodipine, simvastatin, or lovastatin for CYP3A4; (2) theophylline for CYP1A2; (3) S-warfarin for CYP2C9; and (4) desipramine for CYP2D6. If the initial study is positive for inhibition, further studies of other substrates may be useful, representing a range of substrates based on the likelihood of coadministration. For example, possible substrates for further study of a CYP3A4 interacting investigational drug might include dihydropyridine calcium channel blockers and triazolobenzodiazepines, or for a CYP2D6 inhibiting investigational drug might include metoprolol. If the initial study is negative with the most sensitive substrates, it can be presumed that less sensitive substrates will also be unaffected.

2. Investigational Drug as Substrate

In testing an investigational drug for the possibility that its metabolism is inhibited or induced (i.e., as a substrate), selection of the interacting drugs should be based on in vitro or other metabolism studies identifying the enzyme systems that metabolize the drug. The choice of interacting drug should then be based on known, important inhibitors of the pathway under investigation. For example, if the investigational drug is shown to be metabolized by CYP3A4 and the contribution of this enzyme to the overall elimination of this drug is substantial, the choice of inhibitor and inducer could be ketoconazole and rifampin, respectively, because of the substantial effects of these interacting drugs on CYP3A4 metabolism (i.e., they are the most sensitive in identifying an effect of interest). If the study results are negative, then absence of a clinically important drug-drug interaction for the metabolic pathway could be claimed. If the clinical study of the most potent specific inhibitor/inducer is positive and the sponsor wishes to claim lack of an interaction between the test drug and other less potent specific inhibitors, or give advice on dosage adjustment, further clinical studies would generally be recommended. Certain approved drugs are not optimal selections as the interacting drug. For example, cimetidine is not considered an optimal choice to represent drugs inhibiting a given pathway because its inhibition affects multiple metabolic pathways as well as certain drug transporters.

D. Route of Administration

The route of administration chosen for a metabolic drug-drug interaction study is important. For an investigational agent used as either an interacting drug or substrate, the route of administration should generally be the one planned for in product labeling. When multiple routes are being developed, the necessity for doing metabolic drug-drug interaction studies by all routes should be based on the expected mechanism of interaction and the similarity of corresponding concentration-time profiles for parent and metabolites. If only oral dosage forms will be marketed, studies with an intravenous formulation would not usually be needed, although information from oral and intravenous dosings may be useful in discerning the relative contributions of alterations in absorption and/or presystemic clearance to the overall effect observed for a drug interaction. Sometimes certain routes of administration can reduce the utility of information from a study. For example, an intravenous study would not reveal an interaction for any substrate that exhibits a high extraction ratio or for a low hepatic extraction drugs where intestinal CYP3A4 activity markedly alters bioavailability. For an approved agent used either as a substrate or interacting drug, the route of administration will depend on available marketed formulations, which in most instances will be oral.

E. Dose Selection

For both a substrate (investigational drug or approved drug) and interacting drug (investigational drug or approved drug), testing should maximize the possibility of finding an interaction. For this reason, the maximum planned or approved dose and shortest dosing interval of the interacting drug (as inhibitors or inducers) should be used. Doses smaller than those to be used clinically may be needed for substrates on safety grounds and may be more sensitive to the effect of the interacting drug.

F. Endpoints

1. Pharmacokinetic Endpoints

The following measures and parameters are recommended for assessment of the substrate: (1) exposure measures such as AUC, Cmax, time to Cmax (Tmax), and others as appropriate; and (2) pharmacokinetic parameters such as clearance, volumes of distribution, and half-lives. In some cases, these measures may be of interest for the inhibitor or inducer as well, notably where the study is assessing possible interactions between both study drugs. Additional measures may help in steady state studies (e.g., trough concentration (Cmin)) to demonstrate that dosing strategies were adequate to achieve near steady state before and during the interaction. In certain instances, an understanding of the relationship between dose, blood levels, and response may lead to a special interest in certain pharmacokinetic measures and/or parameters. For

example, if a clinical outcome is most closely related to peak concentration (e.g., tachycardia with sympathomimetics), Cmax or another early exposure measure might be most appropriate. Conversely, if the clinical outcome is related more to extent of absorption, AUC would be preferred. The frequency of sampling should be adequate to allow accurate determination of the relevant measures and/or parameters for the parent and metabolites. For the substrate, whether the investigational drug or approved drug, determination of the pharmacokinetics of important active metabolites is important. Because this guidance focuses on metabolic drug-drug interactions, protein binding determinations are considered unnecessary except for data interpretation.

2. Pharmacodynamic Endpoints

Pharmacokinetic measures are usually sufficient for metabolic drug-drug interaction studies, although pharmacodynamic measures can sometimes provide additional useful information. This may occur when a pharmacokinetic/pharmacodynamic relationship for the substrate endpoints of interest is not established or when pharmacodynamic changes do not result solely from pharmacokinetic interactions (e.g, additive cardiovascular effect of quinidine and tricyclic antidepressants). When an approved drug is studied as a substrate, the pharmacodynamic impact of a given change in blood level (Cmax, AUC) caused by an investigational interaction should be known from other interaction studies about the approved drug, although this may not always be the case for older drugs.

G. Sample Size and Statistical Considerations

For both investigational drugs and approved drugs, when used as substrates and/or interacting drugs in drug-drug interaction studies, the desired goal of the analysis is to determine the clinical significance of any increase or decrease in exposure to the substrate in the presence of the interacting drug. Assuming unchanged PK/PD relationships, changes may be evaluated by comparing pharmacokinetic measures of systemic exposure that are most relevant to an understanding of the relationship between dose (exposure) and therapeutic outcome.

Results of drug-drug interaction studies should be reported as 90% confidence intervals about the geometric mean ratio of the observed pharmacokinetic measures with (S+I) and without the interacting drug (S).[3] Confidence intervals provide an estimate of the distribution of the observed systemic exposure measure ratio of S+I versus S alone and convey a probability of the magnitude of the interaction. In contrast, tests of significance are not appropriate because

[3] Schuirmann, D.J., "A Comparison of the Two One-Sided Tests Procedure and the Power Approach for Assessing the Bioequivalence of Average Bioavailability," *J. Pharmacokin. and Biopharm.*, 15:657-80, 1987.

small, consistent systemic exposure differences can be statistically significant ($p < 0.05$) but not clinically relevant.

When a drug-drug interaction is clearly present (e.g., comparisons indicate twofold or greater increments in systemic exposure measures for S+I) the sponsor should be able to provide specific recommendations regarding the clinical significance of the interaction based on what is known about the dose-response and/or PK/PD relationship for either the investigational agent or the approved drugs used in the study. This information should form the basis for reporting study results and for making recommendations in the package insert with respect to either the dose, dosing regimen adjustments, precautions, warnings, or contraindications of either the investigational drug or the approved drug. FDA recognizes that dose-response and/or PK/PD information may sometimes be incomplete or unavailable, especially for an approved drug used as S.

Second, the sponsor may wish to make specific claims in the package insert that no drug-drug interaction is expected. In these instances, the sponsor should be able to recommend specific *no effect* boundaries, or clinical equivalence intervals, for a drug-drug interaction. No effect boundaries define the interval within which a change in a systemic exposure measure is considered not clinically meaningful. There are three approaches to define no effect boundaries.

Approach 1: No effect boundaries can be based on population (group) average dose and/or concentration-response relationships, PK/PD models, and other available information for the substrate drug. If the 90% confidence interval for the systemic exposure measurement in the drug-drug interaction study falls completely within the no effect boundaries, the sponsor may conclude that no clinically significant drug-drug interaction was present.

Approach 2: No effect boundaries may also be based on the concept that a drug-drug interaction study addresses the question of switchability between the substrate given in combination with an interacting drug (test) versus the substrate given alone. Based on this concept, the sponsor may wish to use an individual equivalence criterion to allow scaling of the no effect boundary and to determine other useful information as well. Sponsors who wish to use this approach are encouraged to contact the Office of Clinical Pharmacology and Biopharmaceutics to discuss approaches to study design and data analysis.

Approach 3: In the absence of no effect boundaries defined in (1) or (2) above, a sponsor may use a default no effect boundary of 80-125% for both the investigational drug and the approved drugs used in the study. When the 90% confidence intervals for systemic exposure ratios fall entirely within the equivalence range of 80-125%, standard Agency practice is to conclude that no clinically significant differences are present.

The selection of the number of subjects for a given drug-drug interaction study will depend on how small an effect is clinically important to detect, or rule out, the inter- and intrasubject variability in pharmacokinetic measurements, and possibly other factors or sources of variability not well recognized. In addition, the number of subjects will depend on how the results of the drug-drug interaction study will be used, as described above.

This guidance should not be interpreted by sponsors as generally recommending the inclusion of some number of subjects in a drug-drug interaction study such that the 90% confidence interval for the ratio of pharmacokinetic measurements falls entirely within the no effect boundaries of 80-125%. This approach, however, could be deemed appropriate by a sponsor, after considering the expected outcome of a drug-drug interaction study, the anticipated magnitude of variability in pharmacokinetic measurements, and the desired label claim that no clinically significant drug-drug interaction was present.

V. LABELING

A. Drug Metabolism

All relevant information on the metabolic pathways and metabolites and pharmacokinetic interaction should be included in the CLINICAL PHARMACOLOGY section of the labeling. The consequences of metabolism and interactions should be placed in PRECAUTIONS/WARNINGS, CONTRAINDICATIONS, and DOSAGE AND ADMINISTRATION sections, as appropriate.

B. Metabolic Drug-Drug Interaction Studies

Relevant in vitro and in vivo metabolic drug-drug interaction data describing the drug's effects on substrates and the effects of inhibitors and inducers on the drug should be presented in the DRUG-DRUG INTERACTIONS section of the labeling in the CLINICAL PHARMACOLOGY section, including both positive and important negative findings. The types of studies on which statements are based should be identified ***briefly*** in the labeling. If findings indicate a known or potential interaction of clinical significance, or lack of an important interaction that might have been expected, these should be mentioned briefly in the clinical pharmacology interactions section and described more fully in the interaction section under PRECAUTIONS, with advice on how to adjust treatment placed in WARNINGS/PRECAUTIONS, DOSAGE AND ADMINISTRATION, and CONTRAINDICATIONS, as appropriate. In certain cases, information based on clinical studies not using the labeled drug under investigation can be described with an explanation that similar results may be expected for the labeled drug. For example, a strong inhibitor of

CYP3A4 does not need to be tested with all 3A4 substrates to warn against an interaction. Examples of appropriate labeling language are provided in italics below.

DRUG-DRUG INTERACTIONS, CLINICAL PHARMACOLOGY

- In vivo metabolic drug-drug interaction studies indicate little or no pharmacokinetic effect:

Data from a drug-drug interaction study involving (drug) and (probe drug) in _____ patients/healthy individuals indicate that the PK disposition of (probe drug) is not altered when the drugs are coadministered. This indicates that (drug) does not inhibit CYP3A4 and will not alter the metabolism of drugs metabolized by this enzyme.

- In vivo metabolic drug-drug interaction studies indicate a clinically significant pharmacokinetic interaction:

The effect of (drug) on the pharmacokinetics of (probe drug) has been studied in _____ patients/healthy subjects. The Cmax, AUC, half-life and clearances of (probe drug) increased/decreased by ____% (90% Confidence Interval: ____ to ____ %) in the presence of (drug). This indicates that (drug) can inhibit the metabolism of drugs metabolized by CYP3A4 and can increase blood concentrations of such drugs. (See PRECAUTIONS, WARNINGS, DOSAGE AND ADMINISTRATION, or CONTRAINDICATIONS sections.)

Specific enzymes have been identified as metabolizing the test drug, but no in vivo or in vitro drug interaction studies have been conducted:

In vitro drug metabolism studies reveal that (drug) is a substrate of the CYP_____ enzyme. No in vitro or clinical drug interaction studies have been performed to evaluate interactions. However, based on the in vitro data, blood concentrations of (drug) are expected to increase in the presence of inhibitors of _____ such as _____,_____, or_____.

- Neither in vivo nor in vitro drug-drug interaction studies have been conducted and there is no significant metabolism of the drug:

In vivo or in vitro drug-drug interaction studies have not been conducted. The drug interaction potential resulting in changes of PK of (drug) is expected to be low because approximately 90% of the recovered dose of (drug) is excreted in the urine as unchanged drug. However, the role of other pathways of drug elimination, including drug transport systems, is not known. In addition, whether (drug) can inhibit or induce metabolic

enzymes is not known. There is potential for drug interactions mediated via modulation of various CYP enzymes.

- In vitro interaction has been studied but no in vivo studies have been conducted to confirm or refute a finding:

In vitro interaction demonstrated:

In vitro drug interaction studies reveal that the metabolism of (drug) is by CYP3A4 and can be inhibited by the CYP3A4 inhibitor ketoconazole. No clinical studies have been performed to evaluate this finding. Based on the in vitro findings, it is likely that ketoconazole, itraconazole, ritonavir, and other 3A4 inhibitors may lead to substantial increase of (drug) blood concentrations. Refer to PRECAUTIONS, as appropriate.

In vitro interaction demonstrated and the substrate drug has substantial first-pass elimination:

In vitro drug interaction studies reveal that the metabolism of (drug) is by CYP3A4 and can be inhibited by the CYP3A4 inhibitor ketoconazole. No clinical studies have been performed to evaluate this finding. Based on the in vitro findings, it is likely that ketoconazole, itraconazole, ritonavir, grapefruit juice, and other 3A4 inhibitors may lead to substantial increase of (drug) blood concentrations. Refer to PRECAUTIONS, as appropriate.

In vitro interaction not demonstrated:

In vitro drug interaction studies reveal no inhibition of the metabolism of (drug) by the CYP3A4 inhibitor ketoconazole. No clinical studies have been performed to evaluate this finding. However, based on the in vitro findings, a metabolic interaction with ketoconazole, grapefruit juice, and other 3A4 inhibitors is not anticipated. Refer to PRECAUTIONS, as appropriate.

PRECAUTIONS and/or WARNINGS

- An interacting drug causes increased concentrations of the substrate but the administration of both drugs may continue with appropriate dosage adjustment. Results of the studies are described in CLINICAL PHARMACOLOGY, DRUG-DRUG INTERACTIONS, PRECAUTIONS and/or WARNINGS and may state:

Drug_____ /class of drug causes significant increases in concentrations of _____ when coadministered, so that dose of _____ must be adjusted (see DOSAGE AND

ADMINISTRATION). If there is an important interaction, information for patients should point this out also.

- An interacting drug causes increased risk because of increased concentrations of the substrate and the interacting drug should not be used with the substrate. After describing the interaction in the CLINICAL PHARMACOLOGY section, there should be a CONTRAINDICATIONS section and possibly a boxed warning if the risk is serious.

Drug_____ /class of drug can cause significant increases in concentrations of drug_____ when coadministered. The two drugs should not be used together.

- No in vitro or in vivo drug interactions were conducted. These are described in CLINICAL PHARMACOLOGY, DRUG-DRUG INTERACTIONS, PRECAUTIONS and/or WARNINGS and may state:

There is potential for drug interactions mediated via modulation of various CYP enzymes.

DOSAGE AND ADMINISTRATION

- An interacting drug causes increased risk because of increased concentrations of the substrate, but the administration for both drugs may continue with suitable monitoring:

Drug_____/class of drug leads to significant increases in blood concentrations of _____ by _____%. The dose of _____ should be decreased by _____% when the patient is also taking _____. Patients should be closely monitored when taking both drugs.

CONTRAINDICATIONS

- An interacting drug causes increased risk because of increased concentrations of the substrate and should not be coadministered:

Drug_____ /class of drug leads to significant increases in blood concentrations of _____, with potentially serious adverse events. Administration of _____ to patients on drug_____/class of drug is contraindicated.